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I hope that this first draft can be appreciated by all operators of Special Clinical Chemistry, Clinical Pharmacology, Occupational and Forensic Toxicology Laboratories.
I would like to thank to all the authors present in this first edition and I hope that soon many others can be part of the next editorial initiatives.

Stefano Sartori

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SPECIAL CLINICAL CHEMISTRY

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"Oxidative stress in chronic headaches: old and new markers"

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Original Article

Oxidative stress in chronic headaches: old and new markers

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ABSTRACT

Objective: To study the effects of free radicals' overproduction in chronic primary headaches, we have evaluated two oxidative stress biomarkers, 3-nitrotyrosine (3-NT) and 8-hydroxy-deoxyguanosine expressed as ratio *vs* 2-deoxyguanosine (8-OHdG/2-dG). **Methods:** Analyses were done in plasma of three groups of subjects: chronic migraine (CM), chronic tension-type headache (CTTH) and healthy controls (CNT). 3-NT was analyzed by ultraviolet high-performance liquid chromatography (HPLC-UV), and 8-OHdG and 2-dG by two similar HPLC methods with electrochemical and fluorimetric detection, respectively. **Results:** In comparison to CNT group, 3-NT levels in plasma were significantly higher in CM patients whereas in CTTH were in the same range of healthy subjects. Instead, the mean 8-OHdG/2-dG ratios were higher in both chronic headache groups than CNT but without statistical significance (P values 0.46 and 0.1 for CTTH and CM, respectively). **Conclusion:** The higher mean plasmatic levels of 3-NT in CM only, suggest that just in this type of migraine an anomalous production of reactive nitrogen species occurs.

KEY WORDS: 2-Deoxyguanosine, 3-nitrotyrosine, 8-hydroxy-deoxyguanosine, chronic migraine, chronic tension-type headache, headache

INTRODUCTION

Chronic forms of primary headaches include chronic migraine (CM) and chronic tension-type headache (CTTH). Although the International Headache Society (IHS) has defined the criteria for the diagnosis of CM, the optimal definition of this primary headache is still a debating question, particularly when the patients use large amounts of acute drugs that lead to medication overuse [1-4].

The major requirement for the diagnosis of CM include the presence of headache for at least 15 days per month and a history of previous typical migraine attacks, with a portion of current attacks being classified as migraine without aura (MWoA) [3].

The prevalence of CM in the European population is quite high, being estimated in the range of 1.4 to 2.2%. In addition, 3% of episodic migraine sufferers and 14% in clinical cohorts may develop CM every year [5-6]. The social burden imposed by CM is relevant in terms of reduced health-related quality of life, increasing medical costs and limitations of daily living activities [7-8].

Similarly, the IHS diagnostic criteria for CTTH include presence of headache for at least 15 days per month being classified as typical tension-type headache attacks [2]. The prevalence of CTTH is reported to be around 14% of the general population.

Although the pathogenesis of migraine and its chronic process are still under investigation, it has been suggested that an increased level of oxidative stress products and consequent redox imbalance may play a significant role. However, the origin of these products is uncertain; it is possible that, during migraine attacks, an increased production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) may occur [9]. Particularly interesting is the role of nitric oxide (NO), an important mediator of neurogenic cranial vessel inflammatory response generated in endothelial cells. Enhanced endothelial NO release may cause changes in cerebral blood flow that ultimately might result in migraine.

Possibly, the frequency of attacks over times may cause migraine chronicity through an accumulation of RNS and ROS and a progressive deterioration of mitochondrial and neuronal functions. Given the difficulty in direct analysis of ROS and RNS due to their high reactivity and short half-life, previous studies on oxidative stress in headache were mainly based on colorimetric analysis of total oxidant and antioxidant blood capacities and on activities of enzymes involved in redox reactions such superoxide dismutase (SOD), glutathione peroxidase (GPX) and catalase (CAT). With our study, we wanted take a step forward by considering more specific stress markers able to highlight molecular damages resulting from oxidative attacks.

For proteins, one of the most sensitive oxidative biomarkers is 3-nitrotyrosine (3-NT), a nitration product of tyrosine residues mediated by RNS such as peroxynitrite anion ($ONOO^-$) and nitrogen dioxide (NO_2); its determination may be particularly interesting in migraine owing to the implication of NO imbalance already described. Due to the ubiquity of the proteins and their presence in free

form as well as in tissue structures, the nitration of tyrosine induced by RNS was proposed as marker of early stages of the pathogenetic process.

The prolonged action of ROS, particularly of hydroxyl radical, may also progressively induce oxidative alterations on nuclear and mitochondrial DNA, as modified nucleobases such 8-hydroxy-deoxyguanine (8-OHdG), subsequent mutations and breaking of DNA double strand. During DNA repairing process and especially following the degradation of nucleic acids consequent to cell death, the modified nucleoside 8-OHdG is released in extracellular fluids. Thanks to its molecular stability and specificity, the 8-OHdG plasma concentration is one of the most reliable markers of systemic oxidative stress. An increase in its diagnostic sensitivity could be obtained with the contemporary assay of the not-hydroxylated nucleoside 2-deoxyguanosine (2-dG). Calculation of 8-OHdG/2-dG ratio could indeed reflect the real DNA oxidative damage because the 8-OHdG value becomes, in this way, independent to the speed of DNA turnover that could be altered by disease and may be subject-specific [10]. The evaluation of both 3-NT, 8-OHdG and 8-OHdG/2dG ratio in chronic migraine seemed us useful to clarify the free radical formation processes and their pathological effects.

SUBJECTS AND METHODS

Patients

A group of 21 CM (5 males and 16 females, mean age 35 ± 15) and 17 CTTH (8 males and 9 females, mean age 32 ± 9) patients presenting at Headache Center of Neurology Departments in three Italian hospitals (Vicenza, Milan and Asti) were enrolled. The inclusion criteria consisted of a history of chronic headaches lasting one year or more and a diagnosis of CM or CTTH [2-3].Because not responsive to prophylactic drug treatments, all the subjects were under symptomatic therapy only.

A cohort of 44 healthy subjects (22 males and 22 females, mean age 32 ± 11) was used as control group. Subjects with headaches, diabetes, hypertension or other relevant disorders were excluded. After obtaining informed consent, the levels of 3-NT, 8-OHdG and 2dG were measured in the plasma of all enrolled subjects with HPLC methods.

Biological samples

Blood was collected in vacuum sealed tubes containing EDTA and immediately centrifuged at 3500 rpm, for 10 min, at +4°C; plasma samples were frozen at -80°C until analysis. Both standards and plasma samples were processed according to the manufacturer's method for 3-NT and as previously described for 8-OHdG and 2-dG [10].

Chemicals and solutions

All chemicals were of analytical grade and purchased from Sigma-Aldrich (Milan, Italy). 3-NT was analyzed with a HPLC-UV kit from Eureka (Ancona, Italy). 8-OHdG and 2-dG assays were performed by two home-made HPLC methods as previously reported [10]. The cartridges for 8-OHdG and 2-dG solid phase extraction (MF C18 Isolute 50 mg) were purchased from Step-Bio (Bologna, Italy).

Stock solutions for 8-OHdG and 2-dG were prepared in water and kept at -80°C until analysis. One aliquot of each stock solution was adequately diluted, first in water and finally in plasma matrix pooled from donor samples to obtain working spiked solutions ranging from 0.01 to 0.5 and from 100 to 5000 ng/ml for 8-OHdG and 2-dG, respectively.

High-performance liquid chromatography analysis

The HPLC system consisted of a model 307 pump and a model 234 autosampler with 100 µl loop, both from Gilson (Middleton, WI, USA). For 8-OHdG and 2-dG assays, the separations were performed on a C18 column (Waters X-Bridge Shield 250 mm x 4.6 mm internal diameter) packed with 5 µ particles, with a precolumn (10 mm x 4.6 mm internal diameter) packed with the same material. The mobile phase was an aqueous solution of 25 mM formic acid with 7% acetonitrile, flushed at 1 ml/min. 8-OHdG was revealed with an electrochemical detector Coulochem II ESA fitted with a model 5011 high-sensitivity cell (first electrode +0.00 V, second +0.620 V); in a separate run, 2-dG was then analyzed with a fluorimetric detector model 920 Jasco (excitation and emission wavelengths 264 and 340 nm). The analytical column for 3-NT was an Agilent Poroshell 120 EC-C18 (50 mm x 4.6 mm internal diameter) packed with 2.7 µ particles and the UV detector a model 875-UV Jasco (wavelength 232 nm).

Statistical analysis

Stata 9.0 software was used; the non-parametric Wilcoxon rank sum test for unpaired data was employed to compare the medians of cases and controls. A significance level of 5% was always adopted ($P < 0.05$).

RESULTS

The employed HPLC methods allow us to analyze 8-OHdG, 2dG and 3-NT in relative short time and with high specificity, sensitivity and accuracy (Figures 1-2).

Both 8-OHdG and 2-dG did not show statistically significant differences between CNT, CTTH and CM patients, although lower values of 2-dG in CM than CNT were very close to the significance, fixed at 5% level (Tables 1-2). Even when considering the mean values of 8-OHdG/2-dG ratio, only little differences were found between CNT and migrainous patients, with mean ratios higher in both chronic headache groups than in controls (Figure 3). These differences were once again not statistically significant.

Instead, 3-NT main levels in plasma were much higher in CM than CTTH and controls (Tables 1-2, Figure 4) and the statistical analysis showed full significance ($P < 0.01$).

Table 1. Plasma levels of 3-NT, 2-dG, 8-OHdG and 8-OHdG/2-dG ratio in controls(CNT), chronic tension type headache (CTTH) and chronic migraine (CM) groups

	3-NT (ng/ml)		8-OHdG (pg/ml)		2-dG (ng/ml)		8-OHdG/2-dG ratio (pg / ng x10^3)	
	mean	SD	mean	SD	mean	SD	mean	SD
CNT (n = 44)	**17.3**	5.1	**129.1**	73.1	**775.5**	201.4	**154**	91.3
CTTH (n = 17)	**15.8**	8.5	**133.8**	112.6	**639.7**	77.1	**203.4**	188.4
CM (n = 21)	**47.4**	33.8	**132.8**	58.5	**673**	93.5	**208.3**	85.9

Table 2. P values for 3-NT, 2-dG, 8-OHdG and 8-OHdG/2-dG ratio between control (CNT), chronic tension-type headache (CTTH) and chronic migraine (CM) groups

	3-NT (ng/ml)	8-OHdG (pg/ml)	2-dG (ng/ml)	8-OHdG/2-dG ratio (pg / ng x10^3)
CNT *vs* CTTH	0.66	0.91	0.17	0.46
CTTH *vs* CM	**0.01****	0.98	0.48	0.93
CNT *vs* CM	**0.00****	0.86	**0.06***	**0.1***

**significantly different; *different, but not significant

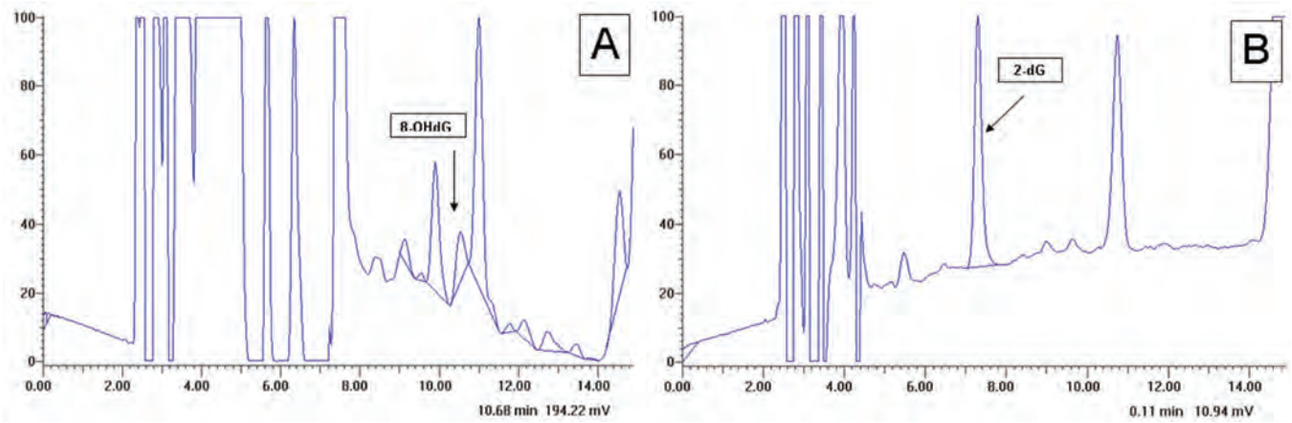

Figure 1. Typical chromatograms of a plasma extract in HPLC conditions for 8-OHdG (**A**) and 2-dG (**B**) respectively. X-axsis shows the chromatographic time in minutes and y-axsis the electrochemical and fluorimetric detector response in relative units.

DISCUSSION

Molecular mechanisms of migraine have not been fully clarified yet and little is known about the role of oxidative stress in the pathogenesis of primary headaches. It was previously reported that strong pro-oxidant species such as thiobarbituric acid reactive substances (TBARS) and nitrate were increased in plasma or urine of migraine patients, with (MWA) and without aura (MWoA), and were associated to platelet membrane alterations and functional abnormalities. These results seemed to indicate that an increased ROS exposition may develop a progressive impairment of platelet function in migraine [11].

Shimomura *et al* [12], showed that platelets activities of antioxidant enzymes such SOD decreased in MWA but not in MWoA and CTTH, suggesting the higher vulnerability to oxidative stress in MWA and its implication in the etiology. Tozzi-Ciancarelli *et al* [13] hypothesize that enhanced endothelium NO and superoxide anion release may cause migraine through changes in cerebral blood flow. To support this theory, they showed that urinary NO stable metabolites (NOx) and TBARS were higher in migraine patients than in a control group [14]; during migraine attack also, NOx and TBARS excretion were higher with respect to the headache-free period. By a preventive non-pharmacological treatment named *biofeedback*, it was possible to decrease migraine attacks influencing NO bioavailability in patients with CM and inducing changes in regional cerebral blood flow mediated by oxygen free radicals that react with NO. The efficacy of *biofeedback* treatment in migraine was even demonstrated in a successive study by determination of NOx, peroxide anion and SOD activity [15].

The association of an increased nitrosative and oxidative stress in migraine attacks was demonstrated also from Yilmaz *et al* [9] with analysis of platelets contents of nitrite and nitrate, as indicators of NO production, and

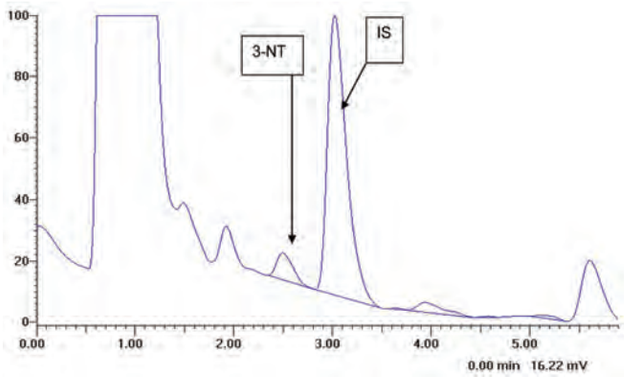

Figure 2. Typical chromatogram for 3-NT and IS in a plasma derivative sample. X-axis shows the chromatographic time in minutes and y-axis the UV-detector response in relative units (0.001 full scale). 3-NT quantification was performed by plasma-matrix calibration and internal standard (IS) correction.

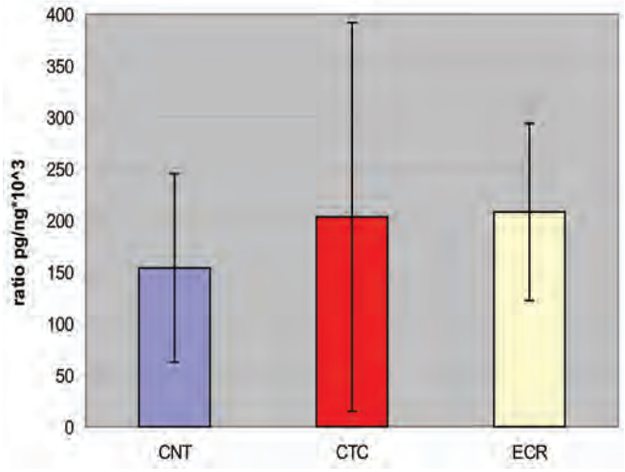

Figure 3. Graphic representation (mean ± SD) of plasma 8-OHdG/2-dG ratio in the three groups.

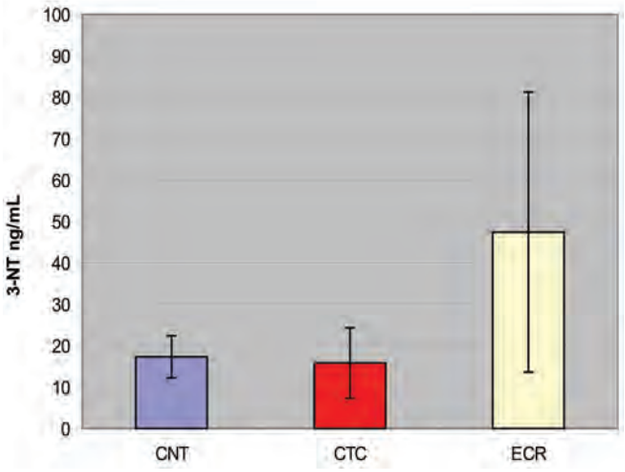

Figure 4. Graphic representation (mean ± SD) of plasma 3-NT levels in the three groups.

malondialdehyde (MDA) and total thiol levels as markers of oxidative status. Particularly the increase of NO activity in platelets during attacks supports the theory that it may play a modulation role on vasodilatation in migraine attacks.

Tuncel *et al* [16], for the first time, hypothesized the pathological role of an impaired mitochondrial oxidative metabolism in migraine. The MDA levels in CM were significantly higher than controls and SOD activity was significantly higher in MWA than in MWoA; instead, no correlation was found between oxidative stress markers and headache attack period. Cordero *et al* [17] remarked the role of mitochondrial dysfunction and oxidative stress in the headache symptoms associated with fibromyalgia. Decrements in coenzyme Q10, catalase and ATP levels in blood mononuclear cells have been found in fibromyalgia; an oral Q10 supplementations showed remarkable improvement in clinical symptoms and headache [18, 19]. More recently, it has been proposed that the observed increase of the elusive amines tyramine, octopamine and synephrine in MWoA and CM could result from a shift of tyrosine metabolism consequent of a mitochondrial damage caused by nitrosative and oxidative stress [20-21].

Studies on migraine and oxidative stress did not always give unequivocal results. Erol *et al* [22], studying the activities of erythrocyte SOD, GPX and CAT in children and adolescents with migraine, found that SOD did not differ between groups, while GPX and CAT were significantly lower in migraine patients than controls. Previously, Bockowski *et al* [23] published that serum and erythrocyte GPX were higher and erythrocyte SOD were decreased in migraine. More recently, Vurucu *et al* [24] investigated the relationship between oxidative stress and chronic daily headache in children finding that erythrocyte SOD, GPX and CAT were all higher in migraine than in control group.

An Indian comparative study between CM and CTTH based on analysis of plasma ferric reducing ability (FRAP) and MDA levels pointed out that tension headaches are not similar to migraines as regards the oxidative stress markers. CM showed highest values of MDA and FRAP while no differences were observed between CTTH and the control group [25].

In recent years, because the strong suggestion of an oxidative imbalance implication, the attention of researchers has focused on molecular pathogenetic alterations in migraine [26]. Ooi *et al* [27], studying the signaling role of NO in neurons of trigeminal ganglia, identified an action site on a triplet of cysteines: this molecular point could be easily a site of oxidative modification mediated by ROS.

So, a tight control of local redox status and NO enviroment could exercise a fine regulation on neural excitability and justify the pathogenetic role of oxidative stress. Even if the oxidative stress may be the cause or the effect of migraine, an antioxidant therapy was introduced in order to re-equilibrate an unbalanced redox status and, as consequence, to reduce severity and frequency of migraine [28]. Dominguez *et al* [29] have already studied organic

extracts of some plants used in folk medicine. Although neither the type of bioactive components or biochemical mechanisms involved have been assessed, this work showed that the extracts can effectively fight the formation of free radicals and TBARS. Chayasirisobhon *et al* [28] studied the benefits of *Pinus radiata* bark extract and vitamin C as treatment for migraine. The responders, who continuously took the bark extract and vitamin C combination for 12 months, experienced ongoing migraine relief with more than 50% reduction of frequency and severity of headaches. Similar conclusions are reported from other Authors who had studied traditional herbal remedies [30], antioxidant [31] and vitamin diet supplementations [32].

Therefore, the results previously reported, taken together, seem to suggest that migraine patients are under an imbalance of redox status due to a continuous nitrosative and oxidative stress generated during the migraine attacks. It was not known if these alterations are present either in CM or in CTTH and if these play a role in the chronic evolution.

In order to investigate the hypothesis of pathogenetic role of oxidative stress in migraine we studied two important markers of damage, 3-NT and 8HdG/2dG in plasma. By-passing the assessment of the effectiveness of oxidative attack and the efficacy of the antioxidant enzymatic and non-enzymatic barrier, it was so possible evaluate the true molecular damages caused by the increased production of ROS and RNS. With full statistical significance, the higher mean levels of 3-NT in plasma of CM patients, seemed to confirm that an abnormality of NO turnover was present in this migraine and that the elevated amount of nitrogen radical may play a role in the chronicity process. In contrast, the demonstration that 3-NT plasma levels in CTTH were in the same range of controls seemed to show that NO metabolism in CTTH was quite normal and that the pathogenesis mechanism differs from CM. These results, although in partial contrast with Van der Schueren *et al* [33], which showed no increments of endothelial NO synthase, are instead in good agreement with other reports [34].

The 8-OHdG/2dG ratio resulted slight altered both in CTTH and CM than in CNT, but without full statistical significance. While the mean concentrations of 8-OHdG were about the same for CNT, CTTH and CM, 2-dG was slightly lower in CTTH and CM than CNT. This could imply a less efficient nucleoside recovery pathway in migraine and a consequent decreased restoration of native nucleotides pool. From that decrease may result a slowdown of DNA repairing systems and the persistence of a higher oxidation damage on nucleotide chains. This higher DNA damage would in fact demonstrated by the increased 8-OHdG/2-dG mean ratios both in CTTH and CM than CNT, but the lack of statistical significance makes impossible to draw conclusions.

Anyway, the elevation of 8-OHdG/2-dG ratios both in CTTH and CM seemed to suggest that while the nitrosative damage characterized mainly CM, DNA oxidation affects both forms of primary headaches. Just because the headache attack is the leading cause of the overproduction of ROS and RNS, the scatter of our data and the probably consequential lack of full statistical significance could be depended on the elapsed time between headache attack and blood collection. We are so planning future studies more standardized respect this time.

Taken together, it was known that there is an imbalance between production and neutralization of free oxygen and nitrogen radicals in chronic migraine. The present data confirm that oxidative stress is a common condition in this pathology, since there is a slight increment of 8-OHdG/2dG ratio both in CM and CTTH compared to controls. However, the pathogenetic mechanism is probably different for CTTH and CM. Indeed, the higher mean plasmatic levels of 3-NT in CM only, strongly suggest that just in this type of migraine an anomalous production of RNS occurs. Further studies in this respect are certainly needed.

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Conflict of Interest: None declared

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CLINICAL PHARMACOLOGY

FLYER

“Determinazione sierica della Metformina con metodo Semplice, Veloce ed Affidabile per il monitoraggio nei pazienti in terapia e nei soggetti intossicati”

"Determinazione sierica della Metformina con metodo Semplice, Veloce e Affidabile per il monitoraggio nei pazienti in terapia e nei soggetti intossicati"

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Istituti Clinici Scientifici Maugeri S.p.A. Società Benefit, IRCSS Pavia

Il Servizio di Tossicologia svolge attività clinica e di ricerca (epidemiologica, clinica e sperimentale) nel campo della tossicologia medica comprendente consultazione tossicologica in urgenza (CAV), tossicovigilanza e allerta in ambito istituzionale e industriale (CNIT), ricerca traslazionale e attività di Medicina di laboratorio-diagnosi di intossicazioni e monitoraggio farmaco-tossicologico (Lab Tossicologia Clinica (SMeL) e sperimentale)

L'obiettivo del Laboratorio di Tossicologia Clinica e Sperimentale è quello di **monitorare i livelli sierici di metformina**, in pazienti in trattamento per il diabete mellito di tipo 2 (insulino-indipendente) in monoterapia e in associazione alle sulfoniluree e/o ad altri farmaci secretagoghi, ai tiazolidinedioni e all'insulina, ed in pazienti intossicati.

Nel 2016 risultano circa **422 milioni le persone affette da diabete mellito di tipo 2**, rispetto ai circa 285 milioni nel 2010 e ai 100 milioni registrati nel 1980 (Lancet, 2016).

La metformina è il farmaco di prima linea nel trattamento del diabete di tipo 2 sia nel paziente sovrappeso che normopeso. Nei pazienti diabetici che non riescono a controllare la glicemia modificando il proprio stile di vita (alimentazione ed esercizio fisico), **la metformina rappresenta il cardine della cura** anche quando è indicato un trattamento combinato con più farmaci antidiabetici (AMD & SID, 2014; AIFA, 2011; Nathan et al., 2009).

Gli **effetti collaterali più comuni**, riferiti al 20% circa dei pazienti all'inizio del trattamento, consistono in disturbi, gastrointestinali, quali nausea, perdita di appetito, sapore metallico e diarrea (Chan et al., 1999). L'acidosi lattica è un effetto collaterale raro ma potenzialmente life-threatening, con un'incidenza di 2-9 casi per 100.000 pazienti/anno (Biradar et al., 2010; Lalau, 2010) e una mortalità complessiva del 50% circa (Chan et al., 1999; Seidowsky et al., 2009; Lalau et al., 1995; Lalau et al., 1999).

In particolare, la tossicità associata ad un accumulo di metformina è caratterizzata tipicamente da una triade di:

- √ **Insufficienza renale acuta**
- √ **Elevate concentrazioni plasmatiche di metformina**
- √ **Grave acidosi lattica**

Il rischio di acidosi lattica aumenta in caso di patologie acute o croniche che possono causare ipossia tessutale (insufficienza respiratoria, scompenso cardiaco acuto, infarto miocardico recente, shock), digiuno o malnutrizione, insufficienza epatica, intossicazione acuta da alcool e alcolismo (AIFA, 2011).

Un recente studio clinico (Vecchio et al., 2014) ha dimostrato che l'acidosi lattica associata ad accumulo di metformina si può verificare:

- a dosaggi inferiori alla dose massima giornaliera raccomandata di 3000 mg
- con esordio tardivo dopo l'inizio della terapia (60,62+/- 45,74 mesi)
- in pazienti (53%) senza preesistenti controindicazioni alla terapia con metformina

Negli ultimi 5 anni su 70 decessi per intossicazione da farmaci registrati dal CAV-CNIT di PAVIA, 35 erano per accumulo di Metformina!!!

Il Laboratorio di Tossicologia Clinica (SMeL) e Sperimentale, nell'ambito dell'attività di Ricerca & Sviluppo, anche sulla base delle esigenze cliniche evidenziate dal CAV, ha messo a punto un **Nuovo Metodo Analitico** per la determinazione della metformina del siero idoneo per il monitoraggio farmacologico nei:

- pazienti in terapia, a supporto del'attività di medici specialisti, (quali ad es. diabetologi, endocrinologi) e dei medici di base

- Pazienti intossicati, a supporto dell'attività di medici d'urgenza, con possibilità di diagnosi differenziale e/o controlli periodici.

Pertanto, sulla base dei numerosi dati epidemiologici e di letteratura, e dell' esperienza della nostra Unità di Tossicologia, pur non essendo ancora mandatorio da parte degli organi regolatori del farmaco, il monitoraggio farmacologico dovrebbe diventare

BUONA PRATICA CLINICA

durante la terapia con Metformina, al fine di prevenirne l'accumulo e la potenziale, conseguente acidosi lattica grave.

"Perché non mettere a disposizione di tutti i Laboratori Clinici del Mondo il NUOVO metodo analitico che permette di migliorare l'aspettativa di vita del paziente ed in alcuni casi salvargli anche la vita?"

A questa domanda ha risposto un'Azienda italiana sensibile a questo specifico problema che ha deciso di
Ingegnerizzare il metodo sotto forma di KIT pronto all'uso

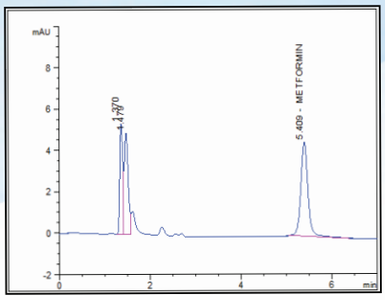
Il kit pronto all'uso è **Semplice** e **Robusto** e comprende tutto il necessario per il trattamento del campione biologico e per l'analisi in cromatografia (HPLC accoppiata ad un detector UV).

Caratteristiche tecnico / analitiche **GARANTITE** dall'eureka kit

eureka Lab Division

L'UNICA Azienda in Italia che Ricerca, Sviluppa e Produce KIT pronti all'uso Sistemi Analitici

✓ **Semplicità**	> purificazione immediata del campione
✓ **Velocità**	> 6 minuti per ottenere un risultato
✓ **Sensibilità**	> LLOD: 0,1 mg/l, LLOQ: 0,2 mg/l
✓ **Accuratezza**	> Accuratezza 3,5%
✓ **Precisione**	> Precisione 3,9 %
✓ **Linearità**	> 0,2 – 100 mg/l
✓ **Recupero dell' analita**	> 98%



Cromatogramma calibratore di Metformina 6 mg/l

Il calibratore liofilo è incluso nel kit, i controlli liofili su due livelli di concentrazione sono disponibili a parte

AZIENDA ULSS 9 TREVISO

Dott. ANDREA ZANARDO
Laboratorio di farmacotossicologia

Il kit prodotto da Eureka Lab Division per il dosaggio della Metformina in HPLC è semplice e veloce.
In poco più di un'ora si possono ottenere i primi risultati di una seduta analitica.
La sensibilità e la riproducibilità sono idonee per il monitoraggio di pazienti in terapia sia su intossicati.
E' proponibile soprattutto per pazienti diabetici con moderata insufficienza renale e per controllare nel tempo i soggetti intossicati.

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POSTER

39° CONGRESSO NAZIONALE LEGA ITALIANA
CONTRO L'EPILESSIA
ROMA 8/10 GIUGNO 2016

"DETERMINAZIONE DELLA CONCENTRAZIONE PLASMATICA DI PERAMPANEL IN HPLC"

DETERMINAZIONE DELLA CONCENTRAZIONE PLASMATICA DI PERAMPANEL IN HPLC

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Obiettivi: il Perampanel (PMP) è un antagonista non competitivo dei recettori del glutammato di tipo AMPA che è stato recentemente approvato come add-on nel trattamento dell'epilessia focale e generalizzata.
Viene metabolizzato per il 98% a livello epatico, principalmente mediante ossidazione seguita da glucuronidazione. Il CYP3A4 è considerato il principale responsabile della idrossilazione del Perampanel e, in minor misura, è coinvolto anche il CYP3A52. Il PMP influisce anche sull'attività di altri isoenzimi di CYP e UGT. Circa il 2% della dose somministrata di PMP è escreta immodificata nelle urine. I valori di eliminazione plasmatica in adulti volontari sani sono di 52-129 h (media 105 h) dopo una singola dose, e di 66-90 h dopo somministrazione di dosi ripetute. È ipotizzabile che dato il suo profilo farmacocinetico il PMP abbia diverse interazioni farmacologiche. Fino ad oggi sono state descritte 12 interazioni farmacocinetiche, fra cui quelle con altri farmaci antiepilettici (vedi tabella).
È noto che il PMP viene ridotto nella sua concentrazione plasmatica (CP) dai farmaci induttori (IAED). Nella pratica clinica può essere importante conoscere la CP del PMP e come questa si modifichi in associazione con altri farmaci antiepilettici (AED) nel singolo paziente.
A nostra conoscenza, ad oggi, non è disponibile un kit commerciale per la determinazione della CP del PMP. Abbiamo, pertanto, messo a punto un metodo per la determinazione della CP del PMP con tecnica cromatografica liquida ad alta risoluzione (HPLC) in fluorescenza.

AED cosomministrato	Influenza di AED sulla concentrazione di Fycompa	Influenza di Fycompa sulla concentrazione AED
Carbamazepina	Riduzione di 2,75 volte	Riduzione <10%
Clobazam	No influenza	Riduzione <10%
Clonazepam	No influenza	No influenza
Lamotrigina	No influenza	Riduzione < 10%
Levetiracetam	Non influenza	No influenza
Oxcarbazepina	Riduzione di 1,9 volte	Aumento 35%
Fenobarbital	No influenza	No influenza
Fenitoina	Riduzione di 1,7 volte	No influenza
Topiramato	Riduzione 19%	No influenza
Ac Valproico	No influenza	Riduzione <10%
Zonisamide	No influenza	No influenza

Tabella: interazioni farmacologiche del PMP

Metodi: in collaborazione con Eureka Lab Division è stato realizzato un kit per il dosaggio fluorimetrico in HPLC del PMP plasmatico.
20 pazienti del Centro Epilessie di Vicenza, che assumevano il farmaco con diverse posologie e con diversi AED associati, sono stati sottoposti a prelievo di sangue prima dell'assunzione della terapia. Il farmaco è stato isolato trattando il plasma con un opportuno deproteinizzante e, dopo l'aggiunta di uno stabilizzante, la soluzione è stata iniettata in HPLC con una corsa di 7 minuti.

Risultati: la performance del nuovo metodo ha prodotto i seguenti risultati: recupero superiore al 98%; sensibilità 0,003 µg/ml; minima concentrazione analizzabile 0,01 µg/ml; linearità 0,01-8 µg/ml; λ_{ex} 290 nm; λ_{em} 430 nm; Gain 0,001 AUFS; Tempo di integrazione 10 secondi; Flusso 1 ml/minuto; RT IS 2,27; RT PMP 4,40 (vedi fig.1 e 2).

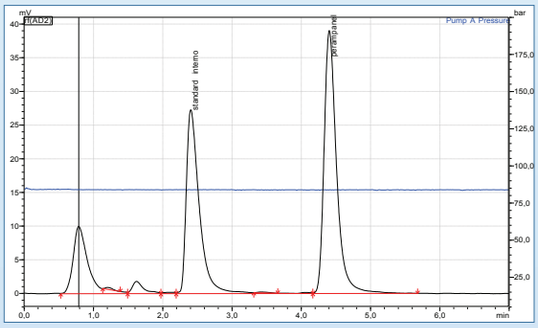

Fig.1: calibratore plasmatico.

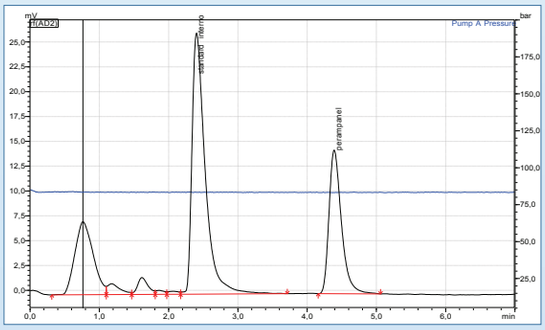

Fig.2: corsa fluorimetrica in paziente in terapia con PMP 10 mg, associato ad acido valproico, rufinamide, lacosamide.

Conclusioni: la CP del PMP in HPLC è facilmente eseguibile, sensibile, riproducibile e utilizzabile per il monitoraggio terapeutico del farmaco nella pratica clinica.

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POSTER

19°Simposio annuale-ELAS-Italia
LIGAND ASSAY 2013
BOLOGNA 25-27 Novembre 2013

"DETERMINAZIONE DEL TOPIRAMATO NEL PLASMA UMANO: CONFRONTO TRA DUE METODI COMMERCIALI"

DETERMINAZIONE DEL TOPIRAMATO NEL PLASMA UMANO: CONFRONTO TRA DUE METODI COMMERCIALI

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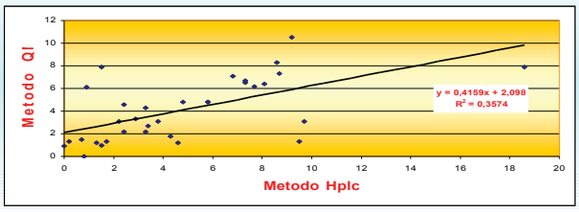
INTRODUZIONE

Nell'ambito del monitoraggio terapeutico dei farmaci antiepilettici vi è sempre maggiore necessità di metodi analitici semplici ed affidabili. Il topiramato è un farmaco anticonvulsivante di seconda generazione che trova applicazione nella cura dell'epilessia e nella cura delle cefalee. Poiché la sua attività farmacologica è significativa già a bassi dosaggi, l'analisi può essere condotta solo con metodi che garantiscano la necessaria sensibilità.

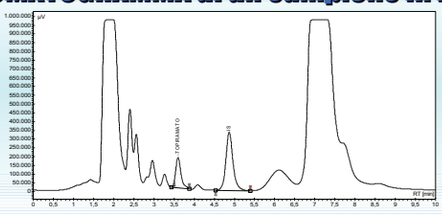
SCOPO

Il nostro studio si è proposto di confrontare le prestazioni di due tra i pochi metodi commerciali attualmente disponibili per la determinazione del topiramato nel plasma umano: un metodo HPLC con derivatizzazione fluorescente (Topiramato plasmatico in fluorimetria, Eureka, Ancona) ed un metodo immuno-turbidimetrico (Thermo Scientific QMS® Topiramate, USA).

CORRELAZIONE (R^2= 0,3574)



CROMATOGRAMMA di un campione in HPLC



RISULTATI

L'analisi turbidimetrica del topiramato è stata condotta subito dopo la raccolta del campione mentre l'analisi HPLC è stata differita di 8-12 settimane ed eseguita in batch su campioni conservati a -20°C fino al momento dell'analisi. Il confronto dei risultati ottenuti è stato eseguito mediante analisi statistica (StatSoft) e costruzione di una retta di regressione. La media dei valori di topiramato ottenuti con metodo turbidimetrico (QMS) è risultata inferiore rispetto al metodo HPLC (4.1 ± 2.7 mg/L vs. 4.8 ± 3.0 mg/L) e la retta di regressione (r^2= 0.35) ha nettamente evidenziato la scarsa correlazione dei valori e l'elevata percentuale di discordanza (18%). Tra i dati discordanti, solo pochi erano prossimi al limite di sensibilità del metodo QMS; per la maggior parte si trattava di coppie in cui i dati del metodo QMS erano molto inferiori rispetto al metodo HPLC. A causa della scarsità del campione disponibile, solo per alcune coppie di valori discordanti il dato HPLC è stato ripetuto e confermato mediante test di recupero e diluizione. Non è stata invece valutata sperimentalmente la ripetibilità del metodo QMS né è stato possibile verificare se i dati discordanti derivassero da pazienti in politerapia.

CONCLUSIONI

I dati ottenuti evidenziano il vantaggio che il metodo HPLC offre rispetto al metodo immunoturbidimetrico, soprattutto a motivo delle sue maggiori sensibilità e specificità. Inoltre, la disponibilità di un kit commerciale HPLC di semplice applicazione nei laboratori di farmaco-tossicologia riduce di molto il vantaggio che spesso deriva dal ricorso ai più semplici ed automatizzabili metodi immunoturbimetrici.
Al fine di meglio descrivere la correlazione tra i metodi studiati, ulteriori approfondimenti si rendono necessari per verificare se i casi di discordanza siano o meno riconducibili alle terapie a basso dosaggio e/o ai casi di politerapia.

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POSTER

20° Simposio annuale-ELAS-Italia
LIGAND ASSAY 2014
BOLOGNA, 24-26 Novembre 2014

"Analisi del topiramato nelle politerapie: quale specificita'?"

ANALISI DEL TOPIRAMATO NELLE POLITERAPIE: QUALE SPECIFICITA'?

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INTRODUZIONE

Le politerapie nel trattamento delle epilessie refrattarie sono state diffusamente praticate in passato con l'intento di ottenere effetti farmacologici sinergici o tossicità meno severe mediante l'impiego di due farmaci antiepilettici (AED) a basso dosaggio al posto di uno soltanto [1]. Più recentemente, dopo l'introduzione di AED di seconda generazione (felbamato, gabapentin, pregabalin, lamotrigina, topiramato, tiagabina, oxcarbazepina, levetiracetam, vigabatrin, zonisamide), il ricorso alla politerapia è stato limitato per importanti ragioni di *compliance* e qualità di vita ma rimane una realtà per molti pazienti epilettici [2]. Il monitoraggio terapeutico degli AED nelle politerapie richiede pertanto l'impiego di metodi analitici affidabili che garantiscano, oltre alla sensibilità, la necessaria specificità.

SCOPO

Abbiamo confrontato i risultati ottenuti con due metodi per la determinazione del topiramato nel plasma umano: un metodo HPLC con derivatizzazione e rivelazione in fluorescenza (Topiramato plasmatico in fluorimetria, Eureka, Ancona) [3] ed un metodo immuno-turbidimetrico competitivo (QMS® Topiramate, Thermo Scientific, USA). Per valutare la specificità analitica dei due metodi, abbiamo analizzato in parallelo i campioni di plasma di 24 pazienti in terapia con topiramato a diversa posologia, tutti in politerapia con altri AED (levetiracetam, vigabatrin, acido valproico, fenobarbital, carbazepina).

RISULTATI

Le concentrazioni di topiramato ottenute con i due metodi sono state analizzate sia mediante rette di regressione che test di Bland-Altman.
La media dei valori ottenuti con metodo immuno-turbidimetrico è risultata superiore rispetto al metodo HPLC (7,3 3,3 mg/L vs. 6,6 3,9 mg/L) e la retta di regressione (formula, $r^2 = 0.66$) ha evidenziato una scarsa correlazione delle coppie (fig.1). La discordanza è risultata nettamente superiore alle basse concentrazioni (da 1 a 5 mg/L, $r^2 = 0.24$) (fig.2) rispetto alle alte (da 5 a 16 mg/L, $r^2 = 0.50$) (fig. 3).
Il test di Bland-Altman ha sottolineato l'esistenza di una tendenziale proporzionalità dell'errore (fig. 4) con sovrastima delle basse concentrazioni da parte del metodo immuno-turbidimetrico, evidenziando pertanto un probabile problema di specificità.
A causa dell'esiguità dei dati, non è stato invece possibile stabilire quale/i tra i farmaci utilizzati in associazione al topiramato possa essere causa di discordanza.

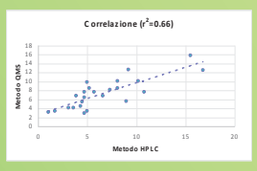

Fig. 1

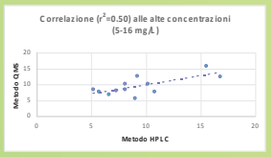

Fig. 3

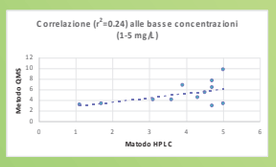

Fig. 2

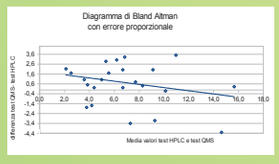

Fig. 4

CONCLUSIONI

In presenza di un difetto di specificità anticorpale, gli immunodosaggi possono dare reazioni crociate in cui l'anticorpo riconosce anche molecole simili a quella da analizzare o molecole diverse ma caratterizzate da epitopi analoghi a quelli dell'analita. Il metodo QMS® Topiramate, utilizzando un solo anticorpo policlonale, come la maggior parte dei metodi competitivi è particolarmente esposto a problemi di specificità ed a cross-reazioni con interferenze generalmente di tipo positivo che sono state confermate dai risultati di questo nostro lavoro. A differenza del metodo immunometrico che basa la propria specificità unicamente sull'interazione antigene-anticorpo e sulla sua univocità, il metodo HPLC si avvale di una tripla specificità: la reazione di derivatizzazione con il fluoroforo, la separazione cromatografica (coppia fase mobile/stazionaria) e la rivelazione in fluorescenza (coppia lambda eccitazione/emissione). La maggiore specificità funzionale dimostrata dal metodo HPLC per il topiramato rispetto al metodo competitivo immuno-turbidimetrico sarà valutata in futuro anche per confronto con immunodosaggi di tipo non competitivo.

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LC/MS

SPECIAL CLINICAL CHEMISTRY

POSTER
presentato presso MSACL, February 21-25, 2016
Palm Springs , CA

"Steroid hormones in serum, a simply, accurate, sensitive and not extractable kit ready to use by LC-MS/MS"

Steroid hormones in serum, a simply, accurate, sensitive and not extractable kit ready to use by LC-MS/MS

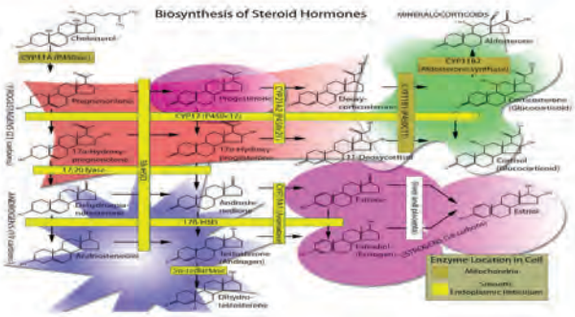
S. Sartori, L. Motti, M. Matteucci, E. Bassotti, G. Alici, L. Falasconi e V. Coniglione
Eureka srl - Lab Division - ITALY

INTRODUCTION

Steroid hormones derived from cholesterol. They are of internal secretion and can cause functional responses in cells located at various distances from its production. The transport into the bloodstream and the achievement of receptors are necessary for the fulfilment of their action. They are able to pass through the cell membrane, causing some changes within it. Steroid metabolism disorder can lead to many endocrine diseases. For this reason, an accurate and sensitive method for the quantification of steroids is necessary.

Nowadays, immunoassay is the most diffuse technique for hormones dosage. However, it often provides non-accurate results due to cross reactivity. In addition, a single parameter test like in immunoassay is very expensive when two or more hormones are required for each sample.

Eureka srl - Lab Division kit is a simply, accurate, sensitive and not extractable kit ready to use by LC-MS/MS able to quantify up to 17 steroids in the same analysis: 17-OH-Progesterone, Dehydroepiandrosterone (DHEA), Dehydroepiandrosterone sulfate (DHEAS), Androstenedione, Cortisol, 11-Deoxycortisol, Corticosterone, Aldosterone, Testosterone, Dihydrotestosterone, Androsterone, Estrone, Estradiol, Pregnenolone,17-OH-Pregnenolone, Progesterone, 11-Deoxycorticosterone.



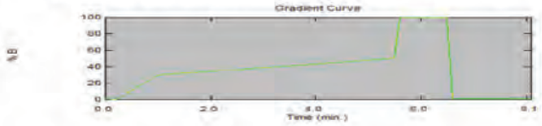
MATERIALS AND METHODS

Sample Preparation

400 µL of human serum (calibrators, controls or samples) were crashed with 1000 µL of Deproteinization Reagent containing isotopic labeled internal standards (see Table 1 for specifications), vortexed and centrifuged for 10 min at 14.000 rpm. 1200 µL of supernatant were transferred, dried and reconstituted with 100 µL of Reconstitution Reagent. 15 µL are injected in to LC-MS/MS. Calibration curve is built by 7 levels calibrators on lyophilized serum matrix (for concentration range see Table 1). Two levels of lyophilized serum matrix controls are available apart. This method can be successfully applied to all medium/high level mass Triple Quad detector coupled with an UHPLC.

LC Method

- LC Gradient: the kit supplies mobile phases A and B.



- Flow: 0.6 mL/min
- Analytical Column: RRHD Eclipse Plus C 18 (50 x 2.1 mm. 1.8 um)
- Column Temperature: 90 °C
- 8 minutes for test (all 17 steroids panel)

MS Method

- Source parameters (Agilent 6460 ESI Jet Stream)
- Gas Temperature 290 °C
- Gas Flow 11 L/min
- Nebulizer 60 psi
- Sheath Gas Heater 400 °C
- Sheath Gas Flow 12 L/min
- Capillary 4500 V

Assay workflow

- Preparation of UHPLC-MS/MS systems: 15 minutes
- Sample Preparation: 3 hours including centrifugation and drying for 50 tests
- Performing the UHPLC-MS/MS assays: 7 hours for 50 tests
- Data processing: 30 minutes for 50 tests

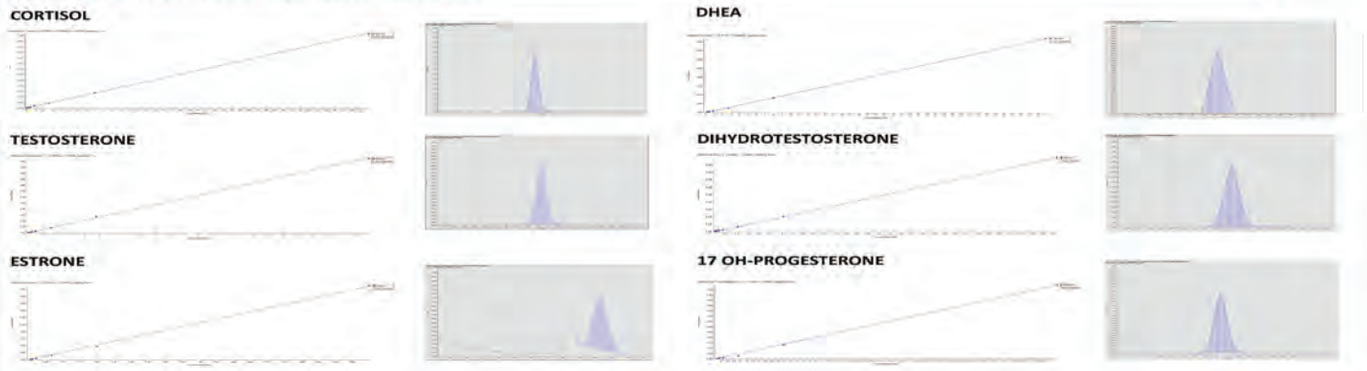
RESULTS AND DISCUSSION

17 steroids are quantified by LC-MS/MS after a simple and fast sample preparation, without the help of any SPE extraction or SPE on LINE. Each injection is very fast, about 8 minutes for one test, including the analytical column regeneration.

Table 1 - Calibration Parameters, LLOQ, Accuracy and Precision

Compound	Isotopic Labeled Internal Standards to be used for calibration (present inside the deproteinization reagent)	Calibration Ranges (ng/mL) given by 7 levels of calibrators	R^2 Regression coefficient	LLOQ (ng/mL) *Agilent 6460 ESI -Jet Stream*	Accuracy intra-day (CV%) Concentration			Precision inter-day (CV%) Concentration		
					Low	Medium	High	Low	Medium	High
17-OH-PROGESTERONE	17 OH progesterone-*d8*	0-6	0.9979 ± 0.0026	0.020	10,6	4,5	3,7	9,0	4,8	3,9
ANDROSTENEDIONE	Testosterone-*d3*	0-4	0.9943 ± 0.0007	0.016	5,7	5,1	4,6	8,2	6,1	6,3
DHEAS	Estradiol-*d5*	0-5000	0.9967 ± 0.0004	15	9,1	8,4	4,0	9,2	6,8	3,3
DHEA	Estradiol-*d5*	0-40	0.9952 ± 0.0003	0.100	10,2	3,3	1,3	11,5	6,1	5,3
TESTOSTERONE	Testosterone-*d3*	0-10	0.9980 ± 0.0007	0.006	4,7	2,1	3,6	5,2	4,8	3,0
CORTISOL	Cortisol-*d4*	0-350	0.9932 ± 0.0064	0.015	2,3	2,0	0,8	9,0	3,9	1,4
CORTICOSTERONE	Aldosterone-*d7*	0-15	0.9968 ± 0.0020	0.036	7,3	8,0	4,4	8,1	7,9	4,1
ALDOSTERONE	Aldosterone-*d7*	0-2.5	0.9970 ± 0.0014	0.020	11,0	4,2	3,5	13,2	7,7	3,2
11-DEOXYCORTISOL	Cortisol-*d4*	0-7	0.9971 ± 0.0004	0.020	11,4	6,0	4,9	12,4	5,0	4,5
DIHYDROTESTOSTERONE	Testosterone-*d3*	0-7	0.9957 ± 0.0034	0.080	8,7	7,0	1,8	11,1	5,9	3,5
ANDROSTERONE	Testosterone-*d3*	0-15	0.9962 ± 0.0026	0.130	14,5	3,1	2,8	17,4	3,8	2,8
ESTRONE	Estradiol-*d5*	0-1.5	0.9972 ± 0.0016	0.010	3,5	2,2	1,4	3,0	3,5	2,4
ESTRADIOL	Estradiol-*d5*	0-2	0.9946 ± 0.0049	0.010	9,3	3,9	3,0	7,8	6,5	2,7
PREGNENOLONE	Pregnenolone-*d4*	0-5	0.9951 ± 0.0051	0.060	12,8	7,8	5,1	11,6	9,1	4,5
17-OH-PREGNENOLONE	Pregnenolone-*d4*	0-6	0.9986 ± 0.0016	0.060	9,3	6,3	1,0	8,0	6,3	2,4
PROGESTERONE	17 OH progesterone-*d8*	0-30	0.9981 ± 0.0001	0.005	5,9	3,9	2,2	5,9	4,1	3,3
11-DEOXYCORTICOSTERONE	Aldosterone-*d7*	0-20	0.9926 ± 0.0086	0.020	10,1	4,5	4,3	11,9	7,6	6,7

CALIBRATION CURVES AND REAL SAMPLES



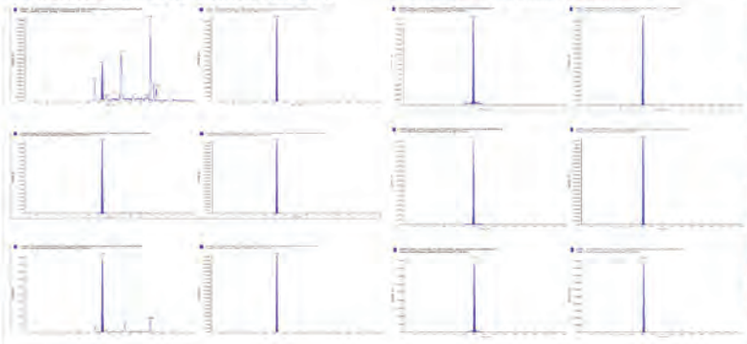
Real Sample
Agilent 6460 ESI Jet Stream

Sample n. 1	Concentration (ng/mL)
Testosterone	0.133
Estradiol	0.040
Cortisol	7.5
Androstenedione	0.093
17 OH progesterone	0.080
DHEAS	105.0
DHEA	0.800
11 Deoxycortisol	0.120
Corticosterone	0.230
Aldosterone	0.040
17 OH Pregnenolone	0.070
Androsterone	0.350
Dihydrotestosterone	0.090
Estrone	0.027
Pregnenolone	0.100
Progesterone	0.600
11 Deoxycorticosterone	0.360

Real samples: critical compounds
5500 Sciex QQQ

Testosterone and its internal standard

DHEA and its internal standard



	TESTOSTERONE (ng/mL)
Sample n. 1	0.11
Sample n. 2	5.85
Sample n. 3	1.06

	DHEA (ng/mL)
Sample n. 1	2.81
Sample n. 2	20.12
Sample n. 3	7.01

CONCLUSIONS

Results show that the method has the following features:

- **Simple**
- **Fast**
- **Accurate**
- **Precise**
- **Sensitive**
- **Specific**
- **Appropriate Calibration Range**

The use of deuterated internal standards has the purpose to correct values for matrix effects improving accuracy.
The use of this kit can overcome immunoassay analytical technology in reliability and speed of execution (with the same sample preparation up to 17 hormones can be detected simultaneously).

In addition to the total solution kit, that includes 17 hormones, other three kits are available:

1. ***Glucocorticoids***: Pregnenolone, 17-OH-Pregnenolone, 17-OH-Progesterone, 11-Deoxycortisol, Cortisol
2. ***Mineralcorticoids***: Pregnenolone, Progesterone, 11-Deoxycorticosterone, Corticosterone, Aldosterone
3. **Sexual hormones**: Pregnenolone, 17-OH-Pregnenolone, Dehydroepiandrosterone (DHEA), Dehydroepiandrosterone sulfate (DHEAS), Androstenedione, Estradiol, Testosterone, Dihydrotestosterone, Androsterone, Estrone

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POSTER

66TH ASMS Conference on Mass Spectrometry and Allied Topics
June 3 - 7, 2018,
San Diego, California

“Fully automated analysis platform for the routine determination of Vitamin D3-25-OH and Vitamin D2-25-OH in plasmatic samples”

SHIMADZU

Fully automated analysis platform for the routine determination of Vitamin D3-25-OH and Vitamin D2-25-OH in plasmatic samples.

Isabel Cabruja[1]; Claudio Ghilardi[1]; Davide Vecchietti[1]; Riccardo Gagliardi[2]; Stephane Moreau[3];
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Overview

We here report a completely automated quantification of Vitamin D in plasma, using a automated sample preparation module online with LC-MS/MS. This method allows routine analysis with reliable data, reduced time, and increased throughput and safety.

1. Introduction

Vitamin D, in addition to parathormone and calcitonin, plays an important role in controlling calcium and phosphor levels in the extracellular fluids. This process maintains skeletal integrity. In the group of Vitamin D, the most important are Vitamin D2 and D3. These molecules need a metabolic process in the liver and in kidneys to be active. This leads to two hydroxylations forming first Vitamin D-25-OH and then Vitamin D-1,25-OH. Vitamin D-25-OH is the circulating molecule. Low levels of 25-OH-D can lead to the resorption of calcium from the bones causing osteoporosis. The fully automated method described here enable the quantification of circulating metabolites 25-OH-Vitamin D2 and D3.

2. Method

The analysis was performed using a fully automatic LCMS preparation Unit (CLAM-2000, Shimadzu) online with LC-MS/MS (Nexera X2 and LCMS-8060, Shimadzu) starting from plasmatic samples kit LC19110 (Eureka). Samples, calibrators, precipitation reagent, and internal standard (Eureka) were directly loaded on CLAM-2000 (Figure 1.).

2-1. Automated sample preparation

CLAM-2000
Automated sample preparation platform
The automated sample preparation method required 40 µL of plasma added to a PTFE filter vial (0.45 µm pore size) previously conditioned with 30 µL methanol. 100 ul of Internal Standard (IS) mixed in Precipitant Reagent (60 uL IS added in 5 mL of Precipitant Reagent) were added. The mixture was stirred for 45 seconds (1200 rpm) and then filtered (by application of vacuum pressure -60 to -65 kPa) for 45 seconds into a collection vial. Finally, 25 µL of the extract was injected into the LC-MS/MS system.

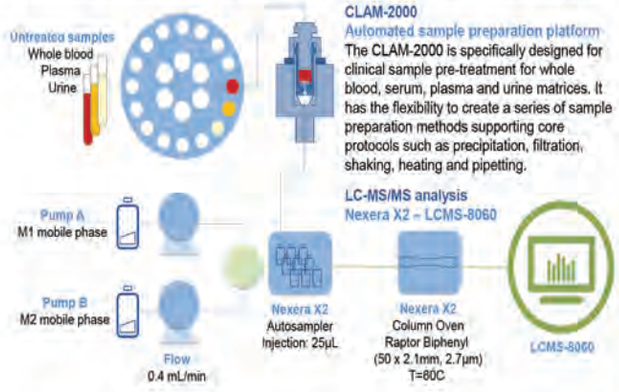


Figure 1. Automated preparation for clinical sample analysis fully integrated with LC-MS/MS.

2-2. MS conditions : LCMS-8060

Ionisation mode : APCI	Interface : 450°C	Drying Gas : 5 L/min (N_2)
Polarity : positive	DL : 250°C	Pause time : 3 msec
Nebulizing Gas : 3 L/min (N_2)	HB : 300°C	Points per peak : > 30

MRM transitions are listed bellow (Table 1.)

Name	MRM 1	MRM 2	MRM 3
25-OH-VitaminD2 (+)	395.4 > 209.2	395.5 > 269.2	413.4 > 355.4
25-OH-VitaminD3 (+)	383.4 > 257.3	383.4 > 211.1	401.4 > 159.1
25-OH-VitaminD3-d6 (+)	407.4 > 159.1	389.4 > 263.1	

Table 1. MRM transitions for each compound.

2-3. Typical chromatogram

The analytes were simultaneously quantified in less than 2.5 min (Figure 2.)

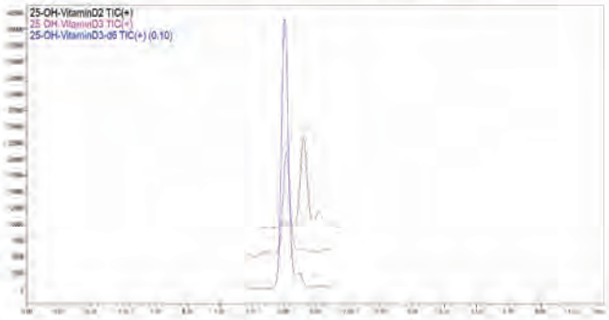


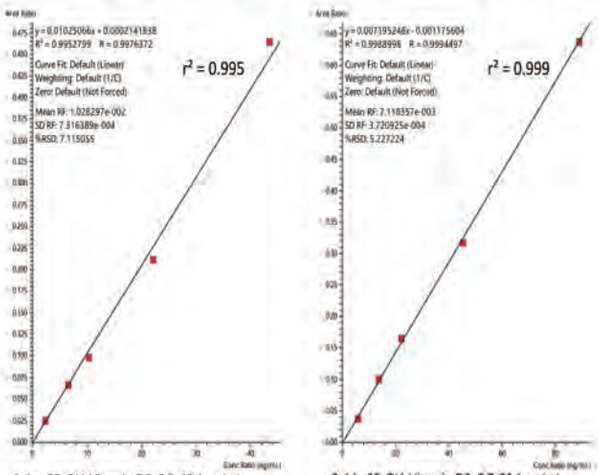
Figure 2. Typical MRM chromatograms for the analysis of 25-OH-Vitamin D2 (6.7 ng/mL), 25-OH-Vitamin D3 (8.7 ng/mL), in plasma control sample.

3. Results

The quantitation of Vitamin D3 and Vitamin D2 in plasmatic samples was performed by LC-MS/MS approach. Usually LC-MS/MS analysis of plasmatic samples require some manual preparation steps for extraction and protein precipitation before the injection. With the aim to reduce the operator involvement, to increase the throughput and the data quality, we completely eliminated the manual sample preparation procedure by the use of a novel automatic preparation unit (CLAM-2000, Shimadzu). We evaluated the linearity range of our method to confirm its compatibility with specific acceptable range. We used a wide range of quantification (5 levels) for each analytes and a good linearity was obtained over the entire range. The intra-day precision (repeatability) of the method was monitored by analyzing two reference samples (Eureka plasmatic controls) spanning from low to high concentration levels and the automated sample preparation provided RSD(%) values complying with the CLSI reference intervals. The accuracy of the method was found to be between 80% and 120% of the target values for all the compounds.

3-1. Calibration curves

The calibration range (Figure 3-1.) was 2.3 - 43.4 ng/mL for 25-OH-Vitamin D2 (3-1.a.) and 5.7 - 89.1 ng/mL for Vitamin D3 (3-1.b.). 25-OH-Vitamin D3-d6 was used as internal standard. For both analytes, the r^2 values of the calibration models were above 0.995. Accuracies are shown in Table 2.



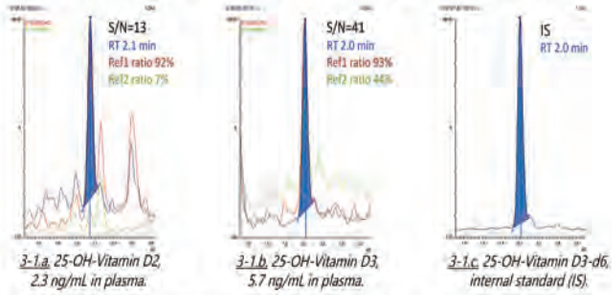
3-1.a. 25-OH-Vitamin D2, 2.3-43.4 ng/mL

3-1.b. 25-OH-Vitamin D3, 5.7-89.1 ng/mL

Figure 3-1. Calibration curves for 25-OH-Vitamin D2 and 25-OH-Vitamin D3.

3-2. Low limits of quantification

The low limits of quantification (LLOQ) in plasma (Figure 3-2.) are 2.3 ng/mL for 25-OH-Vitamin D2 (3-2.a.) and 5.7 ng/mL for 25-OH-Vitamin D3 (3-2.b.). 25-OH-Vitamin D3-d6 was used as internal standard (3-2.c.). The signal to noise ratio (S/N) is above 10 for all LLOQ levels (ASTM).



3-1.a. 25-OH-Vitamin D2, 2.3 ng/mL in plasma.

3-1.b. 25-OH-Vitamin D3, 5.7 ng/mL in plasma.

3-1.c. 25-OH-Vitamin D3-d6, internal standard (IS).

Figure 3-2. MRM chromatograms, at LLOQ levels in plasma, and internal standard.

3-4. Performance Evaluation

Analytical performance of the method was monitored using calibrators and quality controls (QC) provided in plasmatic samples kit LC19110 (Eureka). A wide range of quantification (5 levels) for each analytes was evaluated and the method a good linearity over the entire range. The intra-day precision (repeatability) of the method was confirmed by analyzing two QC samples spanning from low to high concentration levels. Accuracies of calibrators (Table 2.) and QC samples (Tables 3. and 4.) were comprised between 85 and 115% for all analytes, and RSD values (n=8 intra-day) were bellow 10% for both levels of control samples (Tables 3. and 4.).

25-OH-Vitamin D2		25-OH-Vitamin D3	
Conc. (ng/mL)	Accuracy (%)	Conc. (ng/mL)	Accuracy (%)
2.3	108.9	5.7	93.7
6.5	100.2	13.7	104.2
10.3	92.8	22.1	104.4
22.0	93.7	45.2	98.0
43.4	104.4	89.1	99.7

Table 2. Calibrators accuracies (%).

Sample	Conc. (ng/mL)	Range (ng/mL)	Acc. (min - max) (%)	RSD intra-day (n=8) (%)
QC 1	6.7	4.7-8.7	90.1 - 119.8	9.7
QC 2	69.2	48.4-90	87.4 - 100.3	5.0

Table 3. 25-OH-Vitamin D2, QC samples accuracies and RSD values (n=8 intra-day).

Sample	Conc. (ng/mL)	Range (ng/mL)	Acc. (min - max) (%)	RSD intra-day (n=8) (%)
QC 1	8.7	6.1-11.3	97.0 - 109.8	4.3
QC 2	67.6	47.3-87.9	104.2 - 117.1	3.7

Table 4. 25-OH-Vitamin D3, QC samples accuracies and RSD values (n=8 intra-day).

4. Conclusion

- **A key barrier limiting adoption of LC-MS/MS in many laboratories is automating the 'patient sample to result' cycle, however in this work the entire process was successfully automated using the CLAM-2000 integrated with a Shimadzu LC-MS/MS.**
- **This fully automated method enable the quantification of Vitamin D, allowing routine analysis with high data quality and precision, with increased throughput and safety.**

POSTER

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“Fully automated analysis platform for the routine determination of homocysteine in plasma samples”

SHIMADZU

Fully automated analysis platform for the routine determination of homocysteine in plasma samples

Claudio Ghilardi[1]; Davide Vecchietti[1]; Francesco Cante[1]; Riccardo Gagliardi[2]; Stephane Moreau[3];
[1]Shimadzu Italia, Milano, Italy; [2]Eureka Lab Division, Ancona, Italy; [3]Shimadzu Europa GmbH, Duisburg, Germany

Overview

- Elevated homocysteine (HCY) levels are an independent predictor for subsequent cardiovascular mortality or all-cause mortality, and the risks are more pronounced among elderly persons[1].
- To support routine clinical pathology drug monitoring programs, a fully automated sample preparation platform with on-line LC-MS/MS detection for plasma HCY was developed.
- The method delivered a linear calibration curve (concentration range 5.50 to 57.1 umol/L; r2 > 0.99) and QC's were within 85-115% of the target values. Intra-day precision complied with the CLSI reference intervals .

1. Introduction

Homocysteine (HCY) is an essential sulphide amino acid product during the metabolism of methionine to cysteine. A defect on this metabolic pathway or deficiency of certain cofactor can lead to accumulate of HCY causing an increase of cardiovascular risk by activation of monocyte adhesion to the endothelium. For this reason, monitoring HCY levels can be helpful to mitigate the risk of serious and irreversible damage in people with moderate hyperhomocysteinemia. In this work, we report a fully automated method for quantitation of plasma HCY with high throughput and without operator sample preparation.

2. Methods

The analysis of HCY was performed using a fully automated on-line LC-MS preparation platform (CLAM-2000 integrated with a Nexera X2-LCMS8060, Shimadzu Corporation, Japan). A reagent kit (Eureka Lab Division, Italy) was used to provide lyophilized calibrators based on 5 concentration levels and lyophilized controls available as 2 concentration levels.

Reagent kit
Homocysteine (HCY) analysis in plasma
5 reagents
Reducing solution
Buffer solution
Internal standard solution (HCY D8)
Deproteinization solution
Stabilization solution

Reagent kit
Homocysteine (HCY) analysis in plasma
Calibrators and controls
5 levels; 5.5, 10.3, 15.0, 29.8, 57.1 umol/L
Most laboratories report normal homocysteine levels in the blood between 4 and 15 micromoles/liter (µmol/L). Any measurement above 15 is considered high. Optimal homocysteine levels are below 10 to 12. Hyperhomocysteinemia has been classified into moderate, intermediate, and severe types
Moderate (15 to 30 µmol/L)
Intermediate (30 to 100 µmol/L)
Severe (greater than 100 µmol/L)

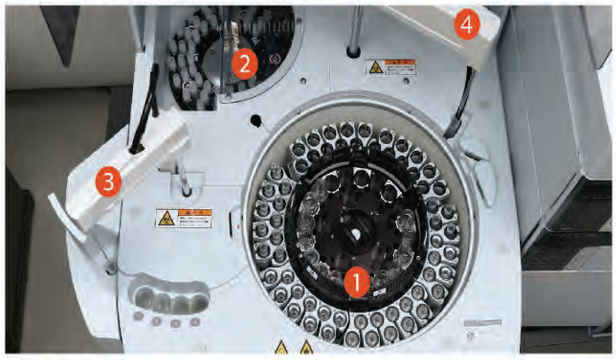
Reagent kit
Homocysteine (HCY) analysis in plasma
Mobile phase
M1 and M2

Figure 1. Schematic representation of the HCY reagent kit used in the automated sample preparation for HCY in human plasma

2.1 Automated sample preparation for LC-MS/MS

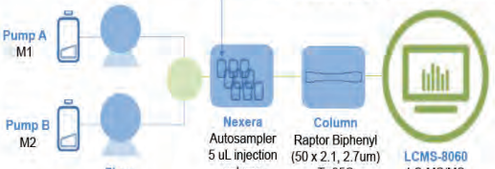
CLAM-2000
Automated sample preparation platform
The CLAM-2000 is specifically designed for clinical sample pre-treatment for whole blood, serum, plasma and urine matrices. It has the flexibility to create a series of sample preparation methods supporting core protocols such as precipitation, filtration, shaking, heating and pipetting.

Clinical Laboratory Automation Module
CLAM-2000
1 Sample and reagent carousel (20 reagent bottles and 60 sample vials); cooler 5-15C
2 Support for sample filtering
3 Sample interface for dispensing samples
4 Reagent interface for transferring reagents



HCY method
Eureka reagent kit loading step
Samples, calibrators, extraction buffer, precipitation reagent, reducing reagent and deuterated internal standard (Eureka) were loaded on CLAM-2000.

CLAM-2000 HCY method
Sample preparation method
The method is fully automated involving 7 steps
Step 1 10 uL of sample were dispensed in filtration
Step 2 Add 20 uL of the deuterated internal standard
Step 3 Add 20 uL reducing reagent
Step 4 Shake and incubate
Step 5 Add 150 uL of precipitation reagent
Step 6 Shake and filter
Step 7 Automated transfer to the Shimadzu LC-MS/MS



Flow
0.8 mL/min
LC Gradient Method
T 0 mins 10% M1
T 2.0 mins 40% M1
T 2.1 mins 90% M1
T 3.0 mins 10% M1

LCMS-8060 MS/MS
HCY
Quant ion 136.1>90.1; CE -12V
Ref ion 136.1>56.2; CE -21V
HCY internal standard (D8)
Quant ion 140.0>59.2; CE -21V
Ref ion 140.0>94.1; CE -12V

Figure 2. Schematic representation of the CLAM-2000 LC-MS/MS method for HCY in human plasma

3. Results

3.1 Human HCY plasma analysis

A series of plasma calibrators were used to evaluate the automated sample preparation protocol (calibration range 5.1- 57.1 umol/L). The calibration range included the normal HCY plasma reference range and was extended to include moderate hyperhomocysteinemia. To assess reproducibility, two plasma controls were repeatedly analysed (low control level and a high control level); n=5.

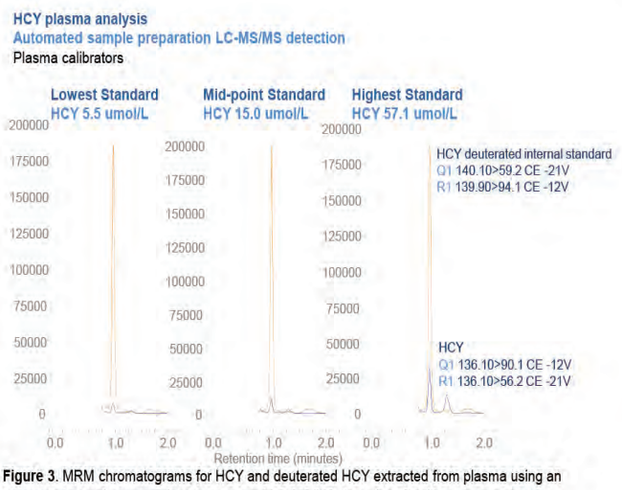


Figure 3. MRM chromatograms for HCY and deuterated HCY extracted from plasma using an automated sample preparation clean up and LC-MS/MS detection. 3 calibrators are shown including the lowest standard at 5.1 umol/L, a mid-point calibrator at 15.0 umol/L and the highest calibrator at 57.1 umol/L.

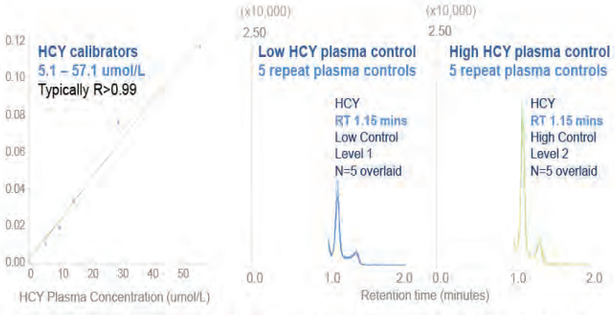


Figure 4. A typical HCY plasma calibration curve over a concentration range of 5.1-57.1umol/L together with overlaid MRM chromatograms for homocysteine (quantitation ion; n=5) for the low control and high control.

3-2. Comparison between an automated approach and a conventional manual sample preparation

In most routine clinical pathology laboratories, automated immunoassay platforms dominate bioanalytical drug assays. LC-MS/MS is increasingly applied to routine forensic and clinical toxicology, it is a highly specific and sensitive technique that can be multiplexed to increase the capability in the development of assays for individual drugs ('laboratory-developed tests' (LDTs) or 'in-house' assays). However, a key barrier limiting its adoption in many laboratories is automating the 'patient sample to result' cycle.

To help accelerate the 'patient sample to result' cycle, a method was developed to automatically quantitate homocysteine in human plasma samples using the CLAM integrated with the LCMS-8060. A reagent kit was used to provide all calibrators, controls and reagents and once the plasma samples were placed in the CLAM no user intervention was required. To assess the performance of the automated platform, the same reagent kit was used manually.

Table 1. Intra-day precision (repeatability) of the automated method was assessed by processing 2 reference samples (Eureka plasmatic controls) control level 1 (low concentration QC) and control level 2 (high concentration QC). The variance (CV%) complied with CLSI reference intervals. The accuracy of the method was found to be between 85% and 115% of the target values for HCY. The results are in good agreement with a manual preparation method.

		Replicates					Mean	Std Dev	% CV	Acceptance Range	Pass
		1.0	2.0	3.0	4.0	5.0					
Control Level 1	Day 1	11.0	11.4	12.9	10.9	11.9	11.6	0.8	6.9	9.6 - 17.9	Yes
	Day 2	13.8	11.2	14.3	13.3	12.0	12.9	1.3	9.9		Yes
	Day 3	14.5	14.6	15.2	14.4	14.1	14.5	0.4	2.8		Yes
Control Level 2	Day 1	25.8	26.0	26.2	29.3	30.0	27.5	2.0	7.4	22.6 - 42.4	Yes
	Day 2	37.4	38.9	38.9	38.3	39.8	38.7	0.9	2.3		Yes
	Day 3	35.0	33.0	34.7	33.4	33.5	33.9	0.9	2.5		Yes

4. Conclusions

- This work presents the first fully automated sample preparation and analysis of HCY in plasma by LC-MS/MS and takes a significant step towards a simplified LC-MS/MS analysis in routine screening laboratories.
- The CLAM-2000 supports integrated calibrators and quality controls throughout the batch analysis, parallel analysis and sample preparation and can be fully adapted to a range of sample preparation protocols including reagent aliquoting, ISTD addition and extraction for automated LC-MS/MS analysis.
- A reagent kit was used to quantitate HCY using lyophilised calibrator and controls.
- The method delivered linear calibration curves for HCY over the concentration range from 5.1 – 57.1 umol/L.
- CV% values complied with the CLSI reference intervals. The accuracy of the method was found to be between 85% and 115%.

POSTER

Presentato al 24° Simposio Annuale ELAS-ITALIA Ligand Assay 2018,
Bologna 19-21 Novembre 2018

"SVILUPPO E VALUTAZIONE DI UN METODO IN LC-MS/MS PER IL DOSAGGIO DELLE AMMINE BIOGENICHE URINARIE"

Sviluppo e valutazione di un metodo in LC-MS/MS per il dosaggio delle ammine biogeniche urinarie

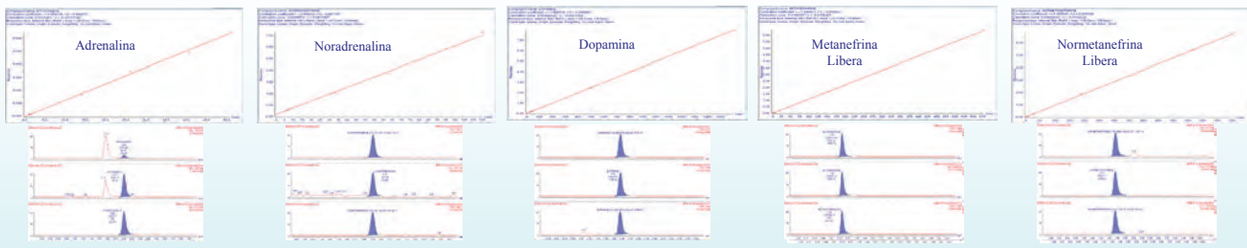
Alessia Francesca Mozzi[1], Chiara Priori[1], Laura Di Felice[1], Elisa Bassotti[2], Davide Ambrogi[3], Stefano Sartori[2], Sergio Bernardini[1]
[1]Policlinico Tor Vergata, Roma [2]Eureka Lab Division, Chiaravalle (AN) [3]Waters Corporation

INTRODUZIONE

Le catecolamine di interesse clinico, adrenalina, noradrenalina e dopamina, sono importanti neurotrasmettitori del Sistema Nervoso Centrale e Periferico: per stimolazione dei recettori adrenergici e dopaminergici sortiscono effetti su diversi sistemi, tra cui quello cardiovascolare. Una loro eccessiva produzione e rilascio si verifica nel caso del feocromocitoma, neoplasia del tessuto cromaffine. Altrettanto importanti sono la metanefrina e la normetanefrina, metaboliti inattivi rispettivamente dell'adrenalina e della noradrenalina. Risulta pertanto fondamentale per la diagnosi il dosaggio delle catecolamine e delle metanefrine urinarie, qui sotto descritte, e plasmatiche. Oggigiorno la determinazione delle catecolamine e delle metanefrine urinarie viene effettuata utilizzando l'HPLC accoppiato ai detector elettrochimico o fluorimetrico. Questo comporta l'utilizzo di due metodi diversi da parte dei Laboratori per la determinazione dei due gruppi di analiti. Il grande vantaggio dell'utilizzo di uno strumento LC-MS/MS è la contemporanea determinazione di catecolamine e metanefrine con un'unica preparativa ed una sola iniezione. Inoltre questo metodo consente di avere sensibilità, accuratezza e precisione migliori dei classici metodi in HPLC.

RISULTATI

Sono state dosate sia le metanefrine che le catecolamine libere con un'unica preparativa usando il metodo Eureka Lab Division. Inoltre sono state quantificate le metanefrine totali usando la stessa preparativa, ma effettuando a priori un'idrolisi enzimatica del campione. Questo perché i classici metodi in HPLC dosano la componente totale per quanto riguarda le metanefrine. Le curve di calibrazione sono state costruite su 5 livelli, usando dei calibratori liofili forniti da Eureka Lab Division e verificandone l'accuratezza con due livelli di controllo.



Ogni analita è stato dosato usando il rispettivo standard interno deuterato. La sensibilità del metodo espressa in LLOQ (Lower Limit Of Quantification) è la seguente: Metanefrina 4.4 µg/L, Normetanefrina 7.3 µg/L, Adrenalina 1.2 µg/L, Noradrenalina 7.2 µg/L, Dopamina 32.5 µg/L. Le accuratezze e le riproducibilità sono sempre inferiori al 6% (per maggiori informazioni si rimanda alla metodica Eureka Lab Division). Per le catecolamine è stata impostata una comparazione fra il dosaggio in HPLC-fluorescenza (HPLC-FLD), HPLC-elettrochimico (HPLC-EC) ed LC-MS/MS.

	Adrenalina			Noradrenalina			Dopamina		
	LC-MS/MS	HPLC-FLD	HPLC-EC	LC-MS/MS	HPLC-FLD	HPLC-EC	LC-MS/MS	HPLC-FLD	HPLC-EC
Range di normalità (µg/24 h)	2.0-22.0	2.0-22.0	0.53-19.5	20-81	20-81	15.30-81.4	40-400	40-400	65.3-402
Campione 1	11	11	9	51	75	61	299	239	365
Campione 2	1.5	3	11	34	54	59	129	152	135
Campione 3	6	5	8	43	57	43	264	247	356
Campione 4	11.9	10.5	10	61	73	65	583	472	538
Campione 5	4	3.1	5	10.7	33.5	26.2	159.1	139.6	157.5
Campione 6	1.9	1.0	7.5	40.8	36.4	48.9	126.7	25.9	24.5
Campione 7	8.6	9.63	10	27.7	47.6	58.7	268.9	177.1	208.7

Per quanto riguarda le metanefrine è stato invece effettuato un confronto fra il dosaggio della frazione libera e della totale, usando sempre la tecnica LC-MS/MS, al fine di verificare se le libere possano essere usate come marker al posto delle totali, dosaggio solitamente effettuato in HPLC. Questo porterebbe ad un duplice vantaggio: 1- idrolisi del campione non necessaria, quindi preparazione del campione più veloce; 2-unica fase preparativa per catecolamine e metanefrine ed ottenimento dei risultati per i 5 analiti di interesse con una sola iniezione.

	Metanefrina		Normetanefrina	
	LC-MS/MS	LC-MS/MS	LC-MS/MS	LC-MS/MS
Frazione	**Libera**	**Totale**	**Libera**	**Totale**
Range di normalità	Uomini < 53.3 µg/24 h Donne < 33.3 µg/24 h	52 - 341 µg/24 h	Uomini < 43.2 µg/24 h Donne < 35.2 µg/24 h	88 - 444 µg/24 h
Campione 1 (donna)	18.7	129.8	33.7	397.4
Campione 2 (uomo)	9.2	39.1	15	91
Campione 3 (uomo)	32.1	210	38.2	284
Campione 4 (donna)	5.6	28	12.7	91.5
Campione 5 (donna)	23.5	149	47.8	463
Campione 6 (uomo)	23.2	121.4	41.5	443.1
Campione 7 (uomo)	17.8	103	23.7	245.7
Campione 8 (donna)	11.1	78.5	30.8	486.8
Campione 9 (uomo)	33.6	115.6	28.9	147.6
Campione 10 (donna)	12.5	126	15.5	410.3

CONCLUSIONI

Si è evidenziato che per tutte e tre le catecolamine i risultati in LC-MS/MS, HPLC-FLD e HPLC-EC sono comparabili in termini di normalità o patologia del campione. Il paziente patologico, borderline o normale secondo una delle tecniche analitiche lo è anche per le altre considerate. Allo stesso modo, il confronto tra la frazione libera e quella totale della metanefrina e della normetanefrina evidenzia una netta corrispondenza tra i valori, confermando che è possibile dosare la sola componente libera per avere una diagnosi adeguata. Questo metodo sviluppato in LC-MS/MS risulta quindi più veloce in termini di preparativa e di refertazione, fornendo risultati affidabili per i 5 analiti di interesse per la diagnosi di feocromocitoma. Rende inoltre possibile dosare anche la 3-metossitiramina, metabolita della dopamina non trattato in questo lavoro. Un metodo similare capace di fornire risultati altrettanto accurati e precisi è disponibile per la matrice plasmatica.

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LC/MS

CLINICAL PHARMACOLOGY

VALIDAZIONE DEL METODO
Istituto di Farmacologia Clinica dell'Università degli studi
"Campus Biomedico di Roma"

"PLASMA LEVEL QUANTIFICATION OF TAMOXIFEN AND ITS ACTIVE METABOLITE ENDOXIFEN BY LIQUID CHROMATOGRAPHIC-TAND MASS SPECTROMETRIC (LC-MS/MS) METHOD"

Plasma level quantification of Tamoxifen and and its active metabolitite Endoxifen by liquid chromatographic–tandem mass spectrometric (LC–MS/MS) method

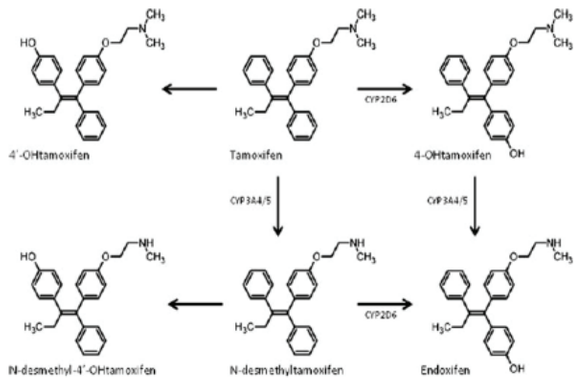
Pierantonio Menna (1), Giulio Alici (2), Elisa Bassotti (2), Mara Matteucci (2)

(1) Drug Sciences and Clinical Pharmacology Unit, University Hospital Campus Bio-Medico of Rome, Italy; (2) Eureka Lab Division / Alifax

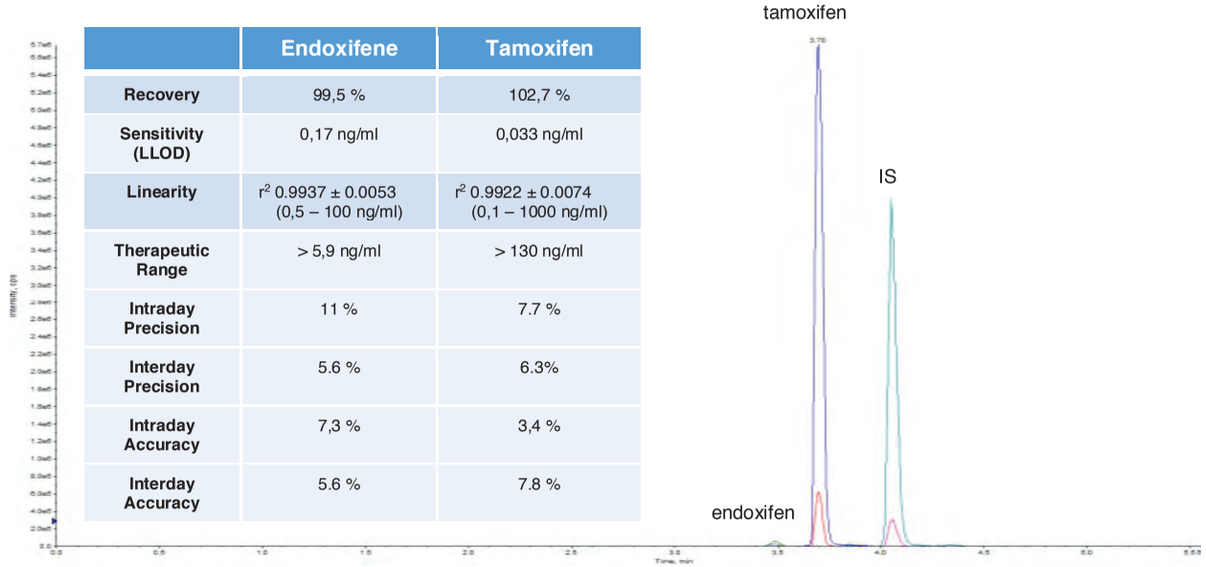
Introduction: Tamoxifen is used as hormone therapy for women with estrogen receptor (ER) positive breast cancer. Tamoxifen binds to ER, preventing proliferation of tumor cells. Cinical response to tamoxifen may depend on the formation of its active metabolite, endoxifen which is generated by cytochrome P450 2D6 isoform. Genetic variants of CYP2D6 may alter response to tamoxifen. Recent data show that patient's genotype only partially explains interindividual differences in plasma endoxifen concentrations. Therefore, therapeutic drug monitoring (TDM) of tamoxifen and endoxifen should be strongly recommended to optimize tamoxifen treatment. In cooperation with Eureka - Lab Division / Alifax we developed a LC–MS/MS method for the determination of tamoxifen and endoxifen in human plasma.

Methods: This method required 100 μL of plasma for single step liquid-liquid extraction and deproteinization. After centrifugation at 13.000 r.p.m. the supernatant is injected into the chromatographic column and analyzed by tandem mass spectrometry. Chromatographic separation was done on a RRHD Eclipse Plus C18 Column (1.8 ìm, 2.1 mm x 50 mm) using 0.1% formic acid/bidistilled water and 0.1% formic acid/acetonitrile as mobile phases. The mass spectrometer worked with electrospray ionization in positive ion mode and multiple reaction monitoring (MRM).

Results:



	Endoxifene	Tamoxifen
Recovery	99,5 %	102,7 %
Sensitivity (LLOD)	0,17 ng/ml	0,033 ng/ml
Linearity	r^2 0.9937 ± 0.0053 (0,5 – 100 ng/ml)	r^2 0.9922 ± 0.0074 (0,1 – 1000 ng/ml)
Therapeutic Range	> 5,9 ng/ml	> 130 ng/ml
Intraday Precision	11 %	7.7 %
Interday Precision	5.6 %	6.3%
Intraday Accuracy	7,3 %	3,4 %
Interday Accuracy	5.6 %	7.8 %



Conclusion: Our method is reliable and robust. It provides a diagnostic tool for monitoring or personalizing tamoxifen treatment. Therapeutic drug monitoring helps to control all factors influencing endoxifen exposure. For example, regular concomitant measurements of tamoxifen and endoxifen concentrations may help to detect variables like patient's poor compliance, defective genetic variants, effects of concomitant drugs.

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POSTER

MSACL - Salisburg 10-14 September 2017

"VOLUMETRIC ABSORPTIVE MICROSAMPLING (VAMS) AND LC-MS/MS ANALYSIS FOR SIMULTANEOUS MONITORING OF 16 ANTIEPILEPTIC DRUGS: WORKFLOW DEVELOPMENT AND VALIDATION"

L3

VOLUMETRIC ABSORPTIVE MICROSAMPLING (VAMS) AND LC-MS/MS ANALYSIS FOR SIMULTANEOUS MONITORING OF 16 ANTIEPILEPTIC DRUGS: WORKFLOW DEVELOPMENT AND VALIDATION

Annachiara D'Urso, Ugo de Grazia

Fondazione IRCCS Istituto Neurologico Carlo Besta

Introduction

Currently, monitoring of antiepileptic drug concentrations in plasma may provide important information to customize the therapy for each patient. Our lab is performing therapeutic drug monitoring (TDM) of anti-epileptics drugs (AEDs) from at least 30 years. This study was designed to evaluate potential application of the newly introduced VAMS sampling technique for TDM of AEDs in routine settings. We develop a liquid chromatography–tandem mass spectrometry (LC–MS/MS) workflow for analysis of VAMS (Mitra™) sample in epileptic patients. In planning this study we considered that VAMS can offer some benefit for patients offering a less painful fingerpicking than a venipuncture; a minimized anxiety (eg: some of epileptic patients experience seizures during blood sampling via venipuncture); a more convenient testing method which can lead to improved adherence/compliance and at-home monitoring so reducing required lab visits. VAMS seems to be more promising than DBS in overcoming issues like HCT bias and sample homogeneity.

Sample preparation

- ✓ Let tips in whole blood from 2 to 4 seconds more than the «red-ding» time
- ✓ Allow tips to dry at least 1H
- ✓ Dip tips in water for 10 seconds
- ✓ Add organic extraction solution
- ✓ Shake in a 96 wells plate for 1 hour at 600 rpm
- ✓ Take surnatants
- ✓ Centrifuge for 5 mins at 15000 g
- ✓ Add acqueous mobile phase
- ✓ Inject 5/10 µl into LC-MS/MS
- ✓ Samples were place in thermostatic autosampler (10°C) and 10µL of sample was injected into the column

Results

- Six point calibration curves demonstrated linear and stable, ranging from a LLOQ lower than the lower limit of therapeutic ranges and a ULOQ higher 2-3 fold than the upper limit of therapeutic ranges, depending on the specific drug.
- CVs% and RSD% for three different levels of quality control are consistently under ±15%.
- Recoveries varies from 86% to 106%, no matrix effect was found for any of the drugs considered (Table 1).
- Samples demonstrated stable (% variation less than 15) at different temperatures for at least 10 days (Figure 1 A and B).
- There were no unexpected endogenous interferences >20% of LLOQ for all compounds (Figure 2).
- Scavenged samples from patients (n=76) had a maximum difference percentage less than ±15% (Figure 3).
- HCT bias was tested for the three levels control at different hematocrit concentrations but no bias (>±15%) was observed (Figure 4).

Tab.1: Results of matrix effect (ME), extraction recovery (RE) and process efficiency (PE)

	%	LEV	PERA	LCM	RUF	ZNS	LTG	TPM	FBM	ETS	OXC	10-MHD	CBZ	CBZ-E	PHT	PHB	PRM
QC LOW	ME	98	102	94	91	93	99	92	93	99	100	97	104	90	86	91	95
	RE	101	98	103	106	94	102	96	105	99	87	104	86	105	100	96	100
	PE	99	99	97	96	88	102	88	97	98	87	101	89	94	86	87	94
QC HIGH	ME	102	103	100	97	104	89	104	99	111	93	105	104	96	98	108	94
	RE	95	100	102	112	97	112	99	100	102	94	105	94	105	101	102	102
	PE	97	103	102	109	102	99	103	100	113	87	110	98	101	99	111	96

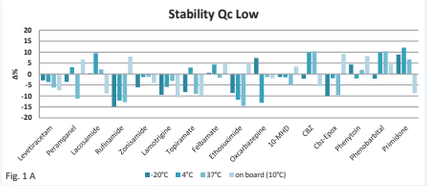


Fig. 1 A

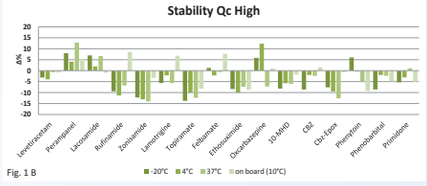


Fig. 1 B

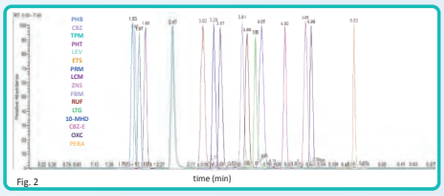
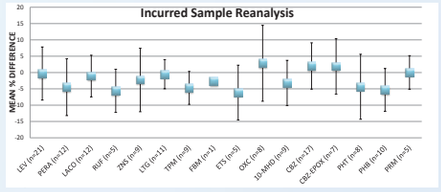


Fig. 2



Hematocrit bias evaluation

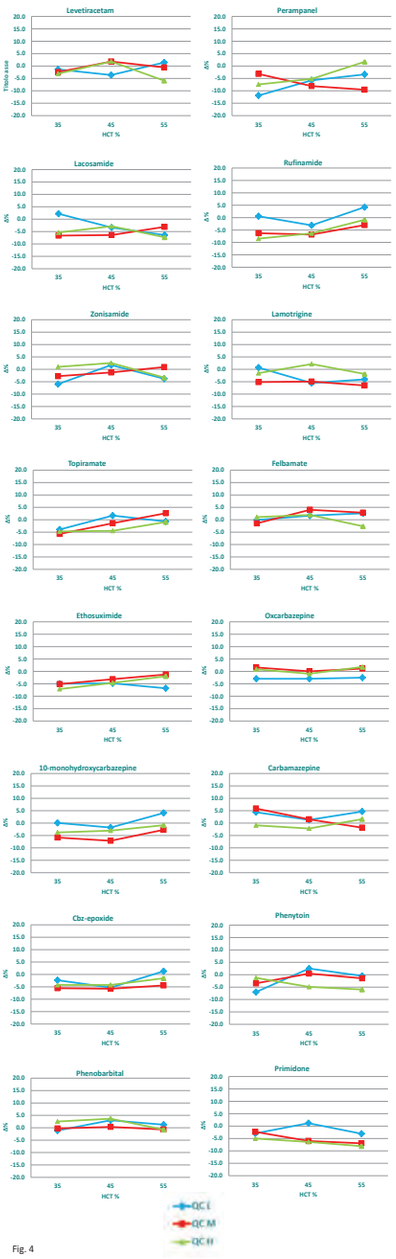


Fig. 4

Conclusions

- VAMS was successfully applied for the first time to the therapeutic drug monitoring of 16 different antiepileptic drugs in epilepsy patients.
- LC–MS/MS workflow for analysis of VAMS sample was developed and validated.
- AED concentrations in whole blood on VAMS device were compared to those in venous blood by routinely used technique with good results.
- Our results established that VAMS is simple, accurate and delivers the benefits of DBS while overcoming the issues of hematocrit and homogeneity and also overcomes issue on stability.

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POSTER

MSACL , February 21-25, 2016 Palm Springs (CA)

" A BRIEF SUMMARY OF FOUR YEARS EXPERIENCE WITH A SIMPLE, ACCURATE, SENSITIVE, READY TO USE KIT FOR SIMULTANEOUS TDM OF 16 AEDS IN SERUM AND PLASMA BY LC-MS/MS"

A brief summary of four years experience with a simple, accurate, sensitive, ready to use kit for simultaneous TDM of 16 AEDs in serum and plasma by LC-MS/MS

Annachiara D'Urso (1), Valentino Coniglione (2), Sara Brambilla (1), Elisa Bassotti (2), Mara Matteucci (2), Luigi Motti (3), Ugo de Grazia (1)

(1) Fondazione IRCCS Istituto Neurologico Carlo Besta, (2) Eureka S.r.l. Lab Division, (3) Alifax Group—diagnostic instrumentation

Introduction

The treatment of the epileptic patients requires a multidisciplinary medical, pharmacological, psychological and social expertise. In this context, therefore, drug therapy and subsequent monitoring play a decisive role in the immediate seizure control and in the medium and long-term prevention of relapse. Currently, thanks to the monitoring of antiepileptic drug concentrations in plasma is possible in many cases to customize the therapy to each individual patients. Our lab is performing therapeutic drug monitoring (TDM) of anti-epileptics drugs (AEDs) from at least 30 years. In the past 4 years we performed 56776 TDM of patients treated for different kind of epilepsy. Among them 27296 were analyzed by chromatography (either HPLC-UV or LC-MS/MS) and 29480 by different immunological methods (on Abbot Architect Plus). Approximately one hundred samples of both group were also retested by LC-MS/MS with a commercial kit from Alifax-Eureka. This study was designed to evaluate the performances of this newly released kit using a validated high performance liquid chromatography–ultraviolet (HPLC-UV) method or immunometric assays as reference method.

Materials and Methods

Two different methods for LC-MS/MS assays were applied using the same kit from Alifax-Eureka "Plasmatic AE in LC-MS – Deuterated Internal Standards": 14 Molecules Method (for ESM, DESM, LMTG, PRM, OXC, CBZ-EPOX, MHD, FELB, RUF, LACO, TPM, CBZ, PB, PHT) and 3 Molecules Method (for LEV, ZNS and *Perampanel*).

Sample Preparation

50 µL of human serum (calibrators, controls or samples) were added to 500 µL of Deproteinization Reagent containing Deuterated Internal Standards, vortexed for 20 seconds and centrifuged for 15 minutes at 13000 rpm after incubation at 2-8°C for 15 minutes. 50 µL of clear supernatant were added to 450 µL of Stabilizing Solution. 10 µL were injected in to LC-MS/MS.

Apparatus

The Thermo Scientific LC-MS/MS apparatus was composed by Trascend TLX-1 HPLC system and TSQ Quantum Access Mass as Mass Spectrometer. The HPLC equipment consisted of an autosampler and a binary pump. The chromatographic separation was perfomed on the Hypersil Gold C18 column (50x2.1 mm, 1,9 µm; Thermo Scientific). The mobile phases were pumped through the column with a flow rate between 0,250 mL/min and 0,500 mL/min according to two main chromatographic methods (Tab 1. and 2.).

MS method

MS analysis was performed using a triple quadrupole mass spectrometer equipped with an Heated Electropray Source Ionization (HESI) operating in both negative and positive-ion mode.

MS setting is illustrated in table 3.

Chromatografic Method A						
Step	Start	Duration (sec)	Flow	Grad	% A	% B
1	0.00	30	0.25	Step	100.0	-
2	0.50	280	0.25	Step	78.0	22.0
3	5.17	90	0.50	Step	-	100.0
4	6.67	120	0.50	Step	100,0	-
5	8.67	100	0.25	Step	100,0	-

Table 1. Chromatografic Conditions for Method A

Chromatografic Method B						
Step	Start	Duration (sec)	Flow	Grad	% A	% B
1	0.00	30	0.40	Step	98.0	2.0
2	0.50	30	0.40	Ramp	-	100.0
3	1.00	90	0.50	Step	-	100.0
4	2.50	90	0.50	Step	98.0	2.0
5	4.00	90	0.40	Step	98.0	2.0

Table 2. Chromatografic Conditions for Method B

Source	Heated Electrospray Ionization (HESI)	
Method	3 Mol	14 Mol
Vaporizer Temp (°C)	350	350
Capillary Temp (°C)	270	300
Aux Gas Pressure (Arb)	20	25
Sheath Gas Pressure (Arb)	40	45
Spray Voltage	Pos 3500	Pos 4000
	Neg 4000	Neg 3500

Table 3. Mass Spectrometer Setting

Results and discussion

- Chromatographic separation: **high specificity**
- Sensitivity: up to **142** times lower than lowest therapeutic values,
  between **1.5** and **25** times hightest than upper therapeutic values.
- Precision: CV_{Intra} **% 5.38 ± 0.77;** CV_{Inter}**% 7.13 ± 1.53**
- Accuracy %: **100.22 % ±8.41**
- Linearity: in averange R^2 **0.995± 0.002**
- Recovery: **more than 98%**
- Stability: samples in Stabilizing Solution are **stable for 2 days at 2-8°C**
- Within **10 minutes per sample**
- Clinical Application: **AED's TDM for more than 50 samples per day**

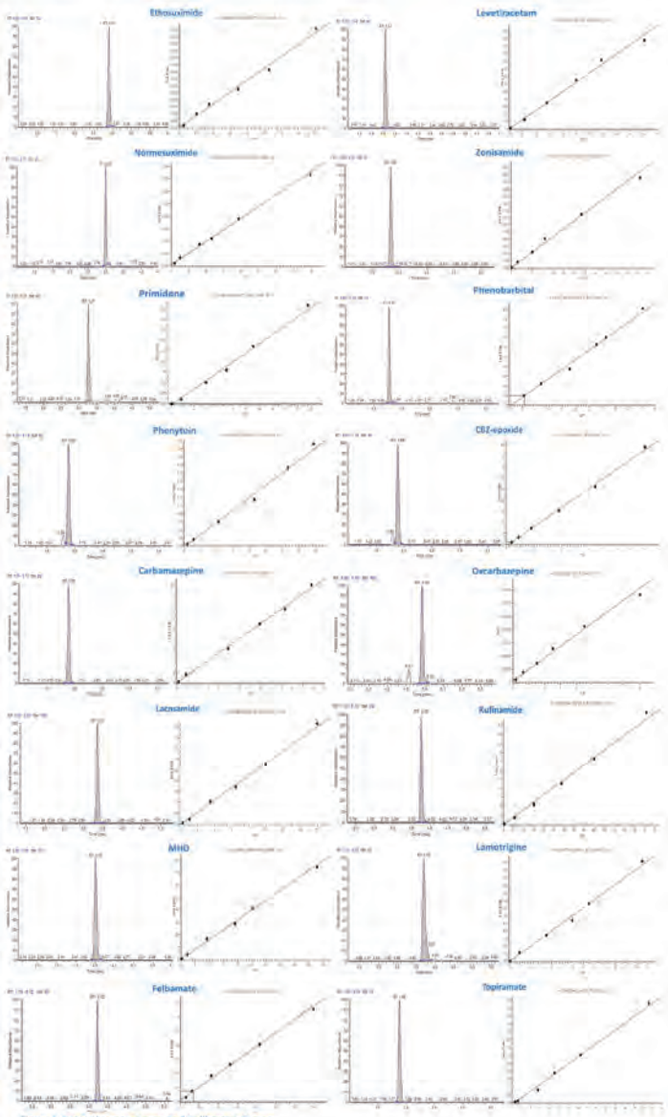

Figure 1. Mass Chromatograms and Calibration Curves

Conclusion

The Alifax-Eureka kit for determination of antiepileptic drugs in plasma/serum, resulted, in our hands, **very easy to use** for sample preparation and subsequent analytical step.

Moreover it is **sensitive**, **specific**, **linear** and **robust** despite the number of different analytes tested. In the first version this kit doesn't include deuterated internal standards. After the introduction of such standards it's strength is noticeably enhanced.

Although we spent some time in kit-instrument tuning, we established **very good chromatographic conditions** allowing us to perform **rapid analysis** with **no matrix effect** and **no analytical interferences.**

In order to increase the test performances we set **two different methods** on the instrument: one dedicated to Levetiracetam and Zonisamide, the other for the remaining drugs.

We tested also online purification but we don't achieve significant improvement so actually we do not include this process in the analysis.

A new study is now ongoing to include Perampanel in our panel of AEDs.

Faster analysis: **LC-MS/MS ~10min per sample**

HPLC ~ 30 min per sample

Immunoassays ~ 25 min per sample

Lowest quantity of sample: **LC-MS/MS 50µL**

HPLC 100µL—200µL

Immunoas. 70µL—80µL

Easy to introduce new compound: **e.g. Perampanel**

Different biological matrices: **e.g. cerebrospinal fluid, urine and whole blood (DBS, microsampling)**

Compound	Parent ion (m/z)	Polarity	Fragment	Deuterated I.S.	RT (min)	R^2 (average)	Cv intra %	Cv inter %	Accurancy %	LOQQ (mg/L)	ULOQ (mg/L)	Therapeutic range (mg/L)
Ethosuximide	139.6	-	/	ESM-d3	2.91	0.992	4,43	5,10	97,1	5,00	150	40-100
Levetiracetam	171.3	+	126.1 69.2	LEV-d6	1.64	0.996	4,36	7,60	96,4	0,07	100	10-37
Normesuximide	190.2	-	161.0 120.1 91.4	DESM-d5	2.99	0.995	5,52	9,20	110,7	0,30	100	10-40
Zonisamide	211.0	-	120.4 119.3	ZNS-d4	1.77	0.992	4,09	4,90	116,6	0,07	100	10-38
Primidone	219.1	+	119.2 115.2 91.2	LACO-d3	3.23	0.994	5,65	5,70	116,8	0,07	30	5-12
Phenobarbital	232.2	-	188.0 82.5 42.5	ZNS-d4	1.84	0.994	6,17	6,30	91,3	1,50	100	10-40
Carbamazepine	237.0	+	194.1 179.1 165.0	LEV-d6	1.89	0.997	4,84	7,40	91,3	0,01	30	6-12
Rufinamide	239.1	+	127.0 107.1 101.9	LACO-d3	3.76	0.996	6,59	7,90	105,5	0,02	100	1-50
Lacosamide	251.5	+	108.1 94.1 74.2	LACO-d3	3.35	0.997	4,59	9,20	96,3	0,03	30	1-10
Oxcarbazepine	253.0	+	236.0 208.0 180.0	LACO-d3	4.93	0.991	4,79	6,50	100,1	0,03	100	0-4
Cbz-epoxide	253.0	+	236.0 210.0 180.0	LACO-d3	4.57	0.998	5,76	6,90	89,3	0,02	100	2-4
Phenytoin	253.3	-	208.1 182.2	ZNS-d4	1.89	0.995	5,84	9,90	94,3	0,30	50	10-20
10-OH-CBZ	255.0	+	237.0 194.0 192.0	LACO-d3	4.00	0.997	5,87	7,90	101,8	0,07	100	3-40
Lamotrigine	256.0	+	210.9 158.9 144.9	LACO-d3	3.70	0.996	5,40	6,50	100,7	0,03	30	3-14
Felbamate	261.1	+	117.2 115.2 91.2	LEV-d6	3.72	0.995	5,68	6,30	95,3	0,10	150	10-80
Topiramate	362.4	-	280.0 96.1 78.2	ZNS-d4	1.88	0.992	6,50	7,80	100,1	0,10	100	2-25

Table 4. Instrumental parameters and statistical values for each analyte

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"A LIQUID CHROMATOGRAPHY – MASS SPECTROMETRY ASSAY FOR DETERMINATION OF PERAMPANEL AND CONCOMITANT ANTIEPILEPTIC DRUGS IN THE PLASMA OF PATIENTS WITH EPILEPSY COMPARED WITH A FLUORESCENT HPLC ASSAY"

A Liquid Chromatography-Mass Spectrometry Assay for Determination of Perampanel and Concomitant Antiepileptic Drugs in the Plasma of Patients With Epilepsy Compared With a Fluorescent HPLC Assay

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Background: Perampanel is a novel noncompetitive selective antagonist at the postsynaptic ionotropic alpha-amino-3-hydroxy-5-methyl-4-isoxazoleproprionic acid (AMPA) glutamate receptor, which is approved as an adjunctive agent for the treatment of partial-onset seizure with or without secondary generalization and for primary generalized tonic-clonic seizure in patients with epilepsy who are at least 12 years of age. Limited information is available about the clinical utility of therapeutic drug monitoring of perampanel and therapeutic ranges are so far not established. Therefore, perampanel titration should be performed especially in case of insufficient success of the drug.

Methods: The authors developed a selective and sensitive LC-MS/MS (liquid chromatography-mass spectrometry) assay to monitor perampanel concentrations in plasma, which was compared to a commercially available high-performance liquid chromatography kit with fluorescent detection. Perampanel and the internal standard were extracted from plasma samples by a simple protein precipitation. The method allows the simultaneous quantification of perampanel and several other antiepileptic drugs (AEDs).

Results: Data were evaluated according to EMA guidelines for bioanalytical method validation. Extraction recovery of perampanel from human plasma was consistently above 98%. No matrix effect was found. Analytical interferences by other AEDs were not observed. The method was linear in the range from 2.5 to 2800 ng/mL. Intra-assay and interassay reproducibility analyses demonstrated accuracy and precision within acceptance criteria. Data collected from 95 patients, given perampanel as their maintenance antiepileptic therapy, showed a very strong correlation between the 2 methods.

Conclusions: The assay allows for highly sensitive and selective quantification of perampanel and concomitant AEDs in patient plasma samples and can be easily implemented in clinical settings. Our findings are in agreement with previously published data in patients comedicated with enzyme inducer AEDs, but seem to indicate a possible interaction in patients treated with the enzyme inhibitor drug valproic acid.

Key Words: perampanel, antiepileptic drug, therapeutic drug monitoring, liquid chromatography mass spectrometry, method validation

(*Ther Drug Monit* 2018;40:477–485)

INTRODUCTION

Perampanel (2-[2-oxo-1-phenyl-5-pyridin-2-y1-1,2 dihydropyridin-3-y1] benzonitrile hydrate) is a novel noncompetitive selective antagonist at the postsynaptic ionotropic alpha-amino-3-hydroxy-5-methyl-4-isoxazoleproprionic acid (AMPA) glutamate receptor.[1] Perampanel (PMP) has a unique mechanism of action for an antiepileptic drug (AED). Studies suggest that AMPA receptor antagonism can lead to inhibition of seizure generation and spread reducing neuronal excitability. Moreover, reduction of neuronal excitability is known as a mechanism preventing neuronal death.[2] The European Medicines Agency (EMA) and the Food and Drug Administration (FDA) approved perampanel (Fycompa, Eisai, Japan) in October 2012 as an adjunctive agent for the treatment of partial-onset seizure with or without secondary generalization in patients at least 12 years of age.

In 2015, a second indication has been approved for perampanel for primary generalized tonic-clonic seizure in patients with epilepsy who are at least 12 years of age.[3] Treatment begins with 2 mg at bedtime and is gradually increased according to clinical response. The recommended dosage range for patients with partial-onset seizure in the absence of enzyme-inducing AEDs is 8–12 mg at bedtime; whereas in patients with primary generalized tonic-clonic seizures, the recommended maintenance dose is 8 mg at bedtime. Adverse events were usually mild or moderate in severity and the most frequent treatment-emergent events reported among perampanel recipients were CNS-related, such as dizziness, somnolence, headache, and fatigue. Administration of

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The authors declare no conflict of interest.

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perampanel results in rapid and complete absorption. The median time to reach peak concentration varies between 0.5 and 2.5 hours in fasting patients and increases up to 3 hours after food intake. Perampanel is substantially protein bound (95%–96%) with only 5% of free perampanel available to be distributed and exert pharmacologic effect.[4,5] Perampanel is metabolized through oxidation, followed by glucuronidation mediated by cytochrome P450 (CYP)3A4/5, CYP1A2, and CYP2B6.[1] The half-life of perampanel is approximately 105 hours in patients who are not concomitantly taking an enzyme-inducing AED. In this condition, the steady state is reached in 2–3 weeks. The elimination of metabolites occurs through urine and feces. About 2% of the dose is excreted unmodified in urine.

Therapeutic drug monitoring (TDM) helps to optimize the dose of AEDs. Only limited information is available about the clinical utility of TDM of perampanel. Given the fact that a reference range is so far not established[6–8] and that the therapeutic range has to be fixed for every patient, TDM titration should be performed for therapy control, dose adjustment, for the control compliance, especially in case of insufficient success of the drug.

In this study, we compared 2 different methods for the quantification of perampanel in human plasma or serum: an existing commercially available HPLC (high-performance liquid chromatography) kit with fluorescent detection, already in use in our laboratories,[9] and another kit for determination of 18 different AEDs (apart from perampanel) based on LC/MS–MS (liquid chromatography-mass spectrometry)[10] to which we added perampanel.[11] These evaluations were performed on plasma samples from patients prescribed perampanel as part of their epilepsy treatment.

TABLE 1. Summary of Patients Data (n = 95)

Gender, n (%)	
Woman	49 (52%)
Man	46 (48%)
Age (yr), median (range)	34.9 (13–67)
Body weight (kg), median (range)	68.8 (38–112)
Perampanel dose (mg)	
Mean (SD)	5.8 (0.24)
Median (range)	6 (2–12)
Perampanel plasma concentration (ng/mL)	
Median (range)	184.0 (35.0–985.0)
Mean (SD)	231.4 (±165.8)
A) Patients comedicated with neutral AEDs (LTG, LEV, ZNS, CLB, CLZ) (n = 17)	313.2 (±232.4)*
B) Patients comedicated with enzyme inducers (CBZ, PB, PHT, PRM, OXC, TPM) (n = 49)	163.2 (±85.2)*
C) Patients comedicated with enzyme inhibitor VPA (n = 15)	359.3 (±199.3)*
D) Patients comedicated with inducers plus VPA (n = 14)	233.5 (±132.7)*

*Mean plasma concentration and SD are expressed in ng/mL.

CBZ, carbazepine; PB, phenobarbital; PHT, phenytoin; PRM, primidone; OXC, oxcarbazepine; LTG, lamotrigine; LEV, levetiracetam; TPM, topiramate; ZNS, zonisamide; CLB, clobazam; and CLZ, clonazepam.

MATERIALS AND METHODS

Processing of Clinical Samples, Calibrators, and QCs

Blood samples collected in vacutainer tubes containing lithium-heparin were obtained from 95 patients given perampanel as their maintenance antiepileptic therapy (56 samples collected at S. Bortolo Hospital, Vicenza and the remaining 39 samples collected at Fondazione Carlo Besta, Milano) (Table 1). Patients, referring to our hospitals for their routine TDM, were selected after at least 14 days assuming the same perampanel dose and all samples were collected in the early morning at least 8 hours after last dose (fasting patients). Samples were centrifuged at 2000*g* for 10 minutes and plasma were separated and stored at −20°C until analysis. Frozen samples were also shared between the 2 laboratories for duplicate measurements. Blank samples were obtained from 6 healthy adult volunteers not treated with AEDs. Commercial samples from external quality assessment schemes (LGC Standards, United Kingdom) were also included in the study.

Informed consent and ethical approval were not necessary because sample collections were obtained from routine clinical TDM.

Reference HPLC Method

Samples for fluorescent HPLC analysis were prepared using a commercial kit (Z79210-Perampanel-FAST; EurekaOne, Chiaravalle, Italy) according to manufacturer instructions. The kit is CE-certified and was developed following Food and Drug Administration Guidance for Industry (FDA-GFI). Kit includes lyophilized calibrator, lyophilized quality controls (QCs), organic deproteinization reagent A (acetonitrile) containing the internal standard, stabilizing solution B (with mobile phase), and organic mobile phase M (water/acetonitrile). The internal standard is patent pending by the manufacturer. Briefly, after reconstitution with 1 mL distilled water, 100 μL of each sample (calibrator, QC samples, or unknown) were dispensed into disposable plastic tubes. After addition of 300 μL of deproteinization solution containing the internal standard, samples were vortex mixed and centrifuged at 14,000*g* for 10 minutes. One hundred microliters of the supernatant were then added to 300 μL of a stabilization buffer. Five microliters of this solution were injected into the HPLC apparatus.

The chromatography apparatus used for the determination of perampanel concentration consisted of a Gilson autosampler (Gilson Autoinjector 234) with a 20-μL loop (Gilson, Villiers-le-Bell, France) and a Shimadzu LC-20AD pump (Shimadzu Italia, Milan, Italy). A Poroshell 120 EC-C18 column (50 × 4.6 mm, 2.7 μm particle) (Agilent Technologies, Santa Clara, CA) was installed on the system. Detection was performed using a fluorescence detector RF-551 (Shimadzu Italia, Milan, Italy) with excitation and

TABLE 2. LC-MS/MS Method Parameters and Partial Validation Data on Other AEDs

Drug	Internal Standard	RT (min)	R^2 (n = 6)	CV% Within-Run (n = 5)			CV% Between-Run (n = 5)		
				L-QC	M-QC	H-QC	L-QC	M-QC	H-QC
LCS	D3-LCS	3.35	0.997	4.9	3.5	2.7	8.1	5.8	2.9
RUF	D3-LCS	3.76	0.996	1.1	9.3	8.1	3.6	6.5	4.5
TPM	D4-ZON	5.20	0.992	4.0	3.7	7.8	6.2	7.8	7.9
ETS	D3-ETS	2.91	0.992	7.7	6.2	1.0	4.9	4.6	3.2
10-OH-OXC	D3-LCS	3.88	0.996	5.3	3.9	4.4	4.2	9.8	6.4
CBZ-E	D3-LCS	4.65	0.998	6.4	3.7	3.9	6.6	5.8	2.6
PHT	D4-ZON	4.20	0.995	8.4	12.0	8.7	3.1	8.8	7.9
FBM	D4-FBM	3.72	0.994	6.4	3.8	5.3	10.2	4.3	5.0
PRM	D3-LCS	3.21	0.993	8.9	5.0	6.2	8.0	9.2	5.8
CBZ	D6-LEV	3.89	0.996	7.2	4.7	1.7	2.8	2.3	6.1
OXC	D3-LCS	5.05	0.991	11.3	9.6	3.0	12.2	11.3	3.4
LTG	D3-LCS	4.21	0.995	7.6	9.6	8.8	11.1	6.1	2.9
LEV	D6-LEV	2.49	0.996	4.3	4.6	7.1	3.4	7.4	6.9
PB	D4-ZON	3.84	0.993	3.9	11.7	6.6	6.1	7.6	6.3
ZNS	D4-ZON	3.58	0.992	3.8	4.0	5.1	2.9	8.6	4.5

Drug	Accuracy Within-Run (n = 5), %			Accuracy Between-Run (n = 5), %			Calibrator Ranges C1–C6* (mg/L)	LOOQ mg/L	ULOQ mg/L	Therapeutic ranges (mg/L)
	L-QC	M-QC	H-QC	L-QC	M-QC	H-QC				
LCS	97.4	99.9	100.4	94.2	100.8	96.7	0.3–11.1	0.03	30	1–10
RUF	101.4	98.6	99.3	100.1	94.7	100.2	1.3–42.4	0.02	100	5–30
TPM	99.4	96.9	97.1	98.7	98.3	98.0	0.6–34.1	0.10	100	2–10
ETS	103.3	93.2	98.4	97.0	96.8	97.3	3.8–165.3	5.00	190	40–100
10-OH-OXC	97.8	98.8	98.4	99.7	95.2	98.2	1.4–51.2	0.07	100	10–35
CBZ-E	99.4	96.6	97.1	96.0	98.4	98.0	0.2–9.6	0.02	100	2–4
PHT	100.2	100.2	100.6	101.5	102.7	109.0	0.9–41.4	0.30	50	10–20
FBM	95.6	98.1	97.9	91.9	98.7	103.2	5.3–130.5	0.10	150	30–80
PRM	100.8	101.7	100.8	100.7	98.6	100.8	0.7–26.3	0.07	30	5–10
CBZ	100.7	98.0	97.8	103.4	101.5	98.1	0.6–25.1	0.01	30	4–12
OXC	95.5	103.3	100.4	95.2	102.6	104.2	0.3–11.0	0.03	100	0–4
LTG	98.2	93.7	98.8	103.2	94.3	97.8	0.5–23.4	0.03	30	3–15
LEV	98.4	96.8	101.8	100.5	101.0	100.3	1.5–52.5	0.07	100	20–40
PB	99.2	93.7	99.7	93.3	98.7	103.4	1.7–69.0	1.50	100	10–40
ZNS	98.6	102.3	103.0	94.0	105.0	100.2	1.1–52.8	0.07	100	10–40

*All curves include the C0 point = 0 mg/L.

emission wavelength set at 290 and 430 nm (other settings were: range 1 and gain 4). The mobile phase was pumped through the column in an isocratic elution mode at 1 mL/min. The retention time of perampanel and retention time of the internal standard were 4.4 and 2.3 minutes, respectively.[9]

Developed LC-MS/MS Method

All chemicals used in this study were a kind gift of Eureka and they are included in a commercial kit for determination of 18 different AEDs by LC-MS/MS (LC05010-Deuterated Antiepileptics kit; EurekaOne) apart from perampanel. Perampanel was a kind gift from Eisai (Eisai Co Ltd, Tokyo, Japan). The kit is CE-certified and was developed following Food and Drug Administration Guidance for Industry (FDA-GFI).

The use of this kit for AEDs monitoring by LC-MS/MS was extensively evaluated in our laboratory. During the last 5 years, we performed monitoring of AEDs concentration in over 15,000 samples from patients undergoing antiepileptic therapy (either as monotherapy or adjunctive therapy). The kit allows the determination in plasma/serum of the following AEDs: carbazepine (CBZ), carbamazepine-epoxide (CBZ-E), oxcarbazepine (OXC), 10-OH-monohydroxycarbazepine (10-OH-OXC), phenytoin (PHT), phenobarbital (PB), primidone (PRM), topiramate (TPM), lamotrigine (LTG), levetiracetam (LEV), felbamate (FBM), zonisamide (ZNS), rufinamide (RUF), lacosamide (LCS), ethosuximide (ETS), metosuximide (MESM), normesuximide (N-DESM), and valproic acid (VPA). Validation data are described by D'Urso et al[10] and precision and accuracy data are shown in Table 2 apart from

MESM, N-DESM, and VPA because of an insufficient number of samples.

The kit contains: mobile phases 1 (water/formic acid) and 2 (acetonitrile/formic acid), organic deproteinization reagent A (acetonitrile) containing a mixture of the deuterated internal standards solutions, 7-point calibration curve (including the C0 = blank matrix calibrator), and 2 QC samples. To accomplish EMA guidelines on bioanalytical methods validation, 2 more QC levels (described in results section) were added for this study. All calibrators and QC samples for

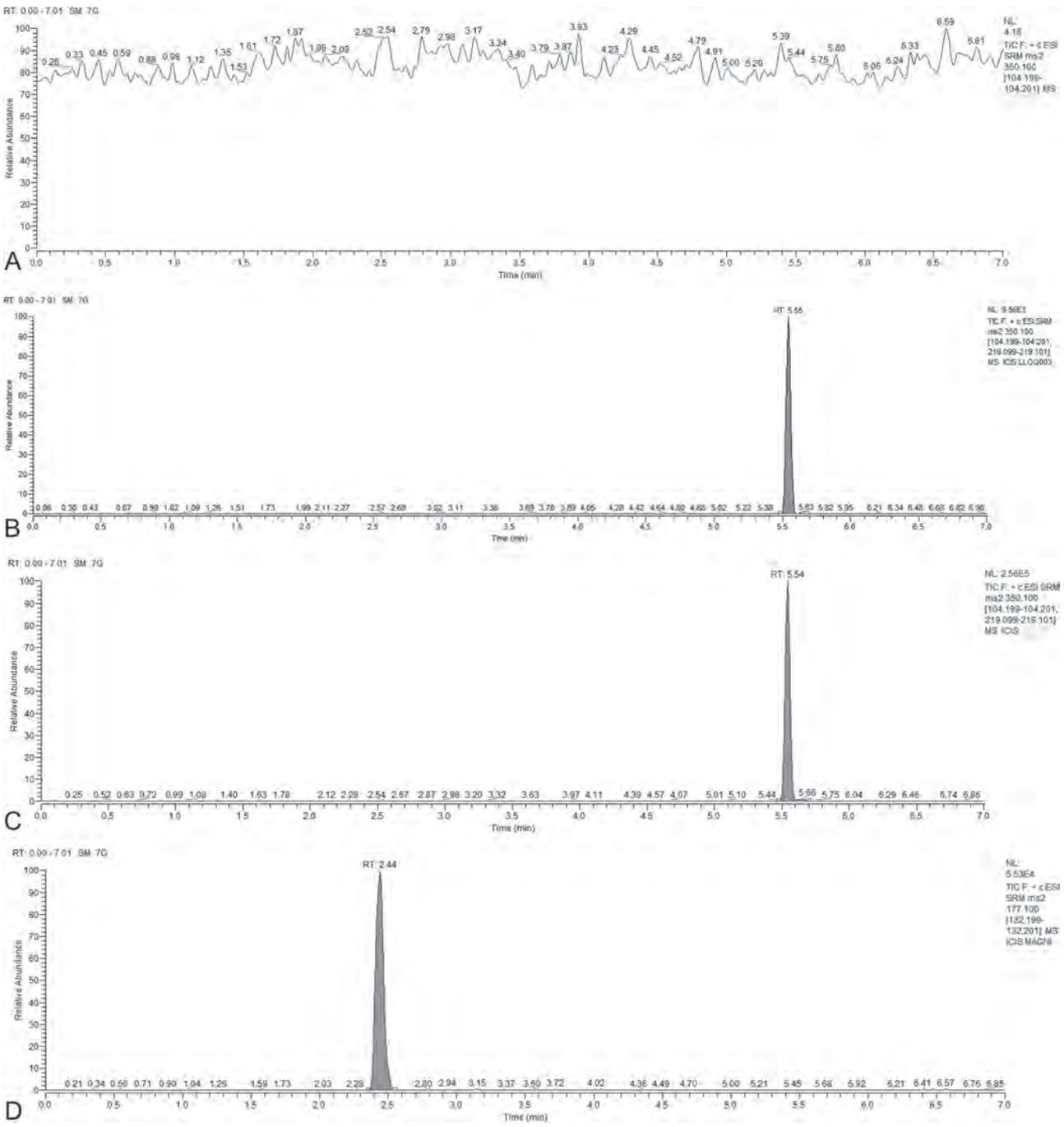

FIGURE 1. LC-MS/MS chromatogram of (A) blank sample; (B) blank sample spiked with perampanel at LLOQ (2.5 ng/mL) (RT = 5.56 minutes); (C) a patient treated with 4 mg/die perampanel (252 ng/mL); (D) the internal standard D6-levetiracetam (RT = 2.44).

LC-MS/MS analysis were prepared spiking kit samples with different concentration of perampanel by kit manufacturer.

Lyophilized calibrators and controls were reconstituted with 1 mL of deionized water obtained from a Milli-Q water purification system (Millipore, Milan, Italy). Fifty microliters of reconstituted calibrators and controls and patient samples were treated with 500 μL of organic deproteinization solution A, containing D6-levetiracetam as the deuterated internal standard and vortex mixed for 20 seconds and centrifuged for 15 minutes at 15,000*g* after incubation at −20°C for 15 minutes. Fifty microliters of clear supernatant were transferred to glass conical vials containing 450 μL of mobile phase 1 and vortex mixed for 20 seconds. Vials were placed in the thermostatic autosampler (10°C) and 5 μL was injected for analysis. A 7-point calibration curve was run with each batch together with QC samples.

LC-MS/MS assays were conducted on a Thermo Scientific tandem mass spectrometer composed by Trascend TLX-1 HPLC system and TSQ Quantum Access Mass as Mass Spectrometer (Thermo Scientific, Milano, Italy). A C18 Hypersil Gold column (50 × 2.1 mm, 1.9 μm particle Thermo-Fisher) was used for chromatographic separation before MS detection.

The initial HPLC conditions had a flow rate of 0.25 mL/min and mobile phase A (100%) was held for 30 seconds to load analytes into the column. At 30 seconds, the mobile phase was stepped to 78:22 (A:B) and held for 280 seconds. At minute 5.12, the flow rate was increased to 0.5 mL/min and mobile phase B switched to 100% and held for 90 seconds. At minute 6.67, the composition of mobile phase was switched in 100% of mobile phase A at the same flow rate and maintained for 120 seconds. At the end, the flow rate was decreased to 0.25 mL/min and maintained for 100 seconds. Total run time was 10.3 minutes per injection to include other AEDs. The retention time of perampanel and retention time of D6-levetiracetam are 5.5 and 2.5 minutes, respectively (Fig. 1). MS analysis was performed using a triple quadrupole mass spectrometer equipped with a Heated Electrospray Source Ionization (H-ESI) operating in positive-ion mode. Precursor and product ion scanning were used to optimize variable MS parameters. Perampanel and D6-levetiracetam (IS) were detected using multiple reaction monitoring (MRM) and the specific transitions are 350.1 > 247.1 m/z for quantifier ion. Two different transitions were considered for qualifier ions: 350.1 > 219.1 and 350.1 > 104.2 m/z.

Collision energies for these transitions were 24, 33, and 34 volts, respectively, whereas tube lens voltage was 88 volts for all transitions.

The internal standard D6-levetiracetam was detected with the specific transition: 177.1 > 132.2 m/z. Data were acquired in selective reaction monitoring mode (SRM), cycle time 0.4 seconds. Ion spray voltage was set at 4000 V and vaporizer temperature at 350°C. Sheath gas and auxiliary gas pressures were set at 45 and 25 arbitrary units, respectively. The capillary temperature was maintained at 300°C. For collision-induced dissociation, high-purity argon was used at a pressure of 1.6 mTorr. Data processing was provided by Thermo Scientific Xcalibur software.

RESULTS

Method Validation

Method validation was performed according to the principles established in the EMA Guideline on Bioanalytical Method Validation[12] including specificity, linearity, intra-assay and interassay imprecision and accuracy, extraction recovery, stability, and incurred sample reanalysis. Matrix effect (ME) was evaluated according to Matuszewski et al.[13]

HPLC Assay

For this method, a partial validation was performed, as required by the guidelines on comparison and validation of a biochemical method,[12] to verify the specifications described by the manufacturer. Intrabatch and interbatch reproducibility was examined by assessing the accuracy and imprecision using 3 QC plasma samples (Low Concentration, 10 ng/mL; Medium Concentration, 200 ng/mL; High Concentration, 1500 ng/mL; 5 replicates/concentration). Measured intrabatch bias was 5.4% for the Low Concentration QC (LowQC) and 3.3% for the High Concentration QC (HighQC) and interbatch bias ranged from 7.8% to 4.1%, respectively. Measured intrabatch CV was 5.8 for LowQC and 3.9 for HighQC; interbatch imprecision was 7.8% for LowQC and 4.7% for HighQC. The calibration curve across 3 assay batches was evaluated and found linear over the concentration range from 10 to 800 ng/mL with acceptable accuracy. The extraction recovery of perampanel from human plasma was 98.1% ± 1.3 for Low QC and 98.3% ± 1.4 for High QC. Extracted samples are stable at least for 2 days at 2–8°C.[9]

LC-MS/MS Assay

Chromatography, Selectivity, and Sensitivity

Chromatographic separation yielded high specificity and sensitivity (Fig. 1). Peaks are sharp and well separated. No endogenous interfering peaks were detected analyzing 6 different blank plasma samples. Analytical interferences by other AEDs were not observed. AEDs evaluated in this study were: CBZ, CBZ-E, OXC, 10-OH-OXC, PHT, PB, PRM, TPM, LTG, LEV, FBM, ZNS, RUF, LCS, VPA.

The lower limit of quantification (LLOQ) was established at 2.5 ng/mL, which is defined as the lowest quantifiable plasma concentration of perampanel with an associated imprecision and inaccuracy ≤20%. The lowest limit of detection was 0.6 ng/mL.

Linearity and Dilution Integrity

Seven-point calibration curve were linear (r = 0.9917) over the concentration range from 50 to 2000 ng/mL (C0 = 0 ng/mL, C1 = 50 ng/mL, C2 = 200 ng/mL, C3 = 500 ng/mL, C4 = 800 ng/mL, C5 = 1300 ng/mL, C6 = 2000 ng/mL). Upper limit of quantification (ULOQ) was 2800 ng/mL.

ULOQ samples of perampanel at 2800 ng/mL for dilution integrity were diluted 1:2 vol/vol with drug-free blank human plasma. The bias and imprecision of 5 determinations were both within ±15%: CV% and accuracy were 3.3% and 103.3%, respectively.

TABLE 3. Imprecision and Accuracy of Perampanel LC-MS/MS Method

	Level	Expected Concentration (ng/mL)	Mean (ng/mL)	SD (ng/mL)	CV %	Accuracy %
Within-run (n = 5)	LLOQ	2.5	2.6	0.3	12.2	102.5
	QClow	7.5	8.1	0.9	11.4	108.6
	QCmedium1	280	291.8	21.3	7.3	104.2
	QCmedium2	900	911.0	26.4	2.9	101.2
	QChigh	1600	1554.6	221.4	14.2	97.2
	ULOQ	2800	2797.3	85.7	3.1	99.9
Between-run (n = 3)	LLOQ	2.5	2.5	0.3	11.7	98.0
	QClow	7.5	7.7	0.7	9.2	102.4
	QCmedium1	280	285.5	14.1	4.9	102.0
	QCmedium2	900	903.9	18.2	2.0	100.4
	QChigh	1600	1624.4	179.6	11.1	101.5
	ULOQ	2800	2879.3	132.8	4.6	102.8

Imprecision, Accuracy Recovery, and ME

Within-run accuracy and imprecision were determined by analyzing 5 replicates per concentration in a single run of LLOQ (2.5 ng/mL), QClow (7.5 ng/mL), QCmedium1 (280 ng/mL), QCmedium2 (900 ng/mL), QChigh (1600 ng/mL), and ULOQ (2800 ng/mL) samples. Between-run accuracy and imprecision were examined on same QC samples from 3 runs analyzed on 3 different days.

Results are shown in Table 3.

Extraction recovery, ME, and process efficiency were investigated on QClow and QChigh samples across 6 different lots of independent drug-free plasma from healthy donors (data are shown in Table 4). The extraction recovery (RE) was calculated by comparing the peak areas of 6 blank samples spiked before extraction at QClow and QChigh concentrations with the peak areas of the analyte in blank samples spiked after extraction at the same concentrations. RE was between 85.0% and 108.5% (mean value for QClow = 100.8% ± 5.5% and for QChigh = 97.5% ± 8.5%). ME was calculated by comparing the peak areas of 6 blank samples extracts spiked after the full sample preparation at the same low and high perampanel concentrations with the peak areas of the analyte in a pure solvent at the same concentrations.

ME was negligible being 97.1% ± 4.5% the mean value for QClow and 106.6% ± 7.2% the value for QChigh. The combined process efficiency is 96.6% ± 8.5% for QClow and 110% ± 16.8% for QChigh.

Data on commercial samples from external quality assessment schemes are reported in Table 5.

Carryover

To evaluate carryover, 5 independent blank samples were injected after samples with high concentrations prepared at the upper limit of analytical measurement range in each run. No carryover was detected for both perampanel and the internal standard.

Stability

Stability was assessed on QC samples. Extracted plasma samples were found to be stable for 48 hours at room temperature, for 10 days either at 4°C or at 10°C (onboard autosampler), and for at least 4 weeks at −20°C. Imprecision and inaccuracy data for both QC levels (QClow and QChigh) used in stability studies were within ±15%.

Incurred Sample Reanalysis

Incurred sample reanalysis was performed on 39 patient samples. Samples were analyzed in 2 different analytical runs; the bias of repeat {[(result 1 − result 2)/mean of result 1 and 2] × 100} was calculated and had to be within 20% in at least

TABLE 4. Matrix Effect (ME), Recovery (RE), and Process Efficiency (PE) data For 2 QC Concentrations (QClow = 7.5 ng/mL and QChigh = 1600 ng/mL) in 6 Different Plasma Lots

	ME (%)		RE (%)		PE (%)	
	QClow	QChigh	QClow	QChigh	QClow	QChigh
Lot n1	100.7	110.0	97.6	85.0	103.2	129.5
Lot n2	96.1	119.7	100.5	89.2	95.6	134.3
Lot n3	99.5	101.3	108.4	100.2	91.8	101.2
Lot n4	95.3	101.4	101.0	101.2	94.3	100.2
Lot n5	89.5	104.2	104.8	104.1	85.4	100.1
Lot n6	101.4	102.7	92.7	105.7	109.4	97.2
Mean	97.1	106.6	100.8	97.5	96.6	110.4
SD	4.5	7.2	5.5	8.5	8.5	16.8

TABLE 5. External Quality Assessment Schemes Results

	Sample	n	Expected value* (ng/mL)	Mean (ng/mL)	CV %	SD
HPLC	AE4 148	6	727.24	640	1.4	9.2
	AE4 149	6	94.7	91	5.7	5.2
	AE4 150	6	49.0	47	5.1	2.4
	AE4 151	6	195	173	4.2	7.2
LC-MS/MS	AE4 148	6	772.9	708.0	3.9	27.4
	AE4 149	6	97.8	105.7	5.7	6.0
	AE4 150	6	47.2	49.2	13.6	6.7
	AE4 151	6	195	204.0	12.7	25.9

*Mean result assigned by vendor according to analysis method (HPLC or LC-MS/MS).

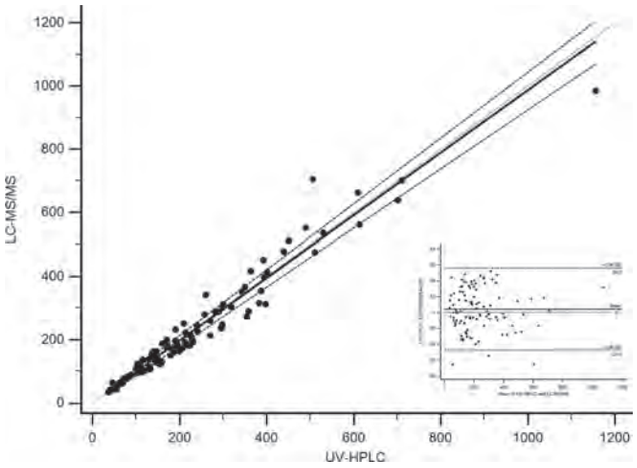

FIGURE 2. Passing-Bablok regression plot of the comparison between the HPLC fluorescence-based assay and the LC-MS/MS method. The regression plot includes the line of identity (slope = 1) (dotted line), the regression line (solid line) with 95% CI (dashed lines) (r = 0.980). In the insert is the Bland-Altman plot; dotted line: reference line for no difference (at 0 ng/mL); solid line: mean percent difference and the 95% limits of agreement of the methods (dashed lines).

67% of the repeats as requested.[12] In 35 of 39 samples, the percentage difference between the concentration of repeat and the original sample was below the threshold limit of 20% specifically recommended by the updated EMA guidelines.

Method Comparison

Cross-validation was performed considering the HPLC fluorescence-based assay as the reference method and LC-MS/MS assay as the tested procedure. Methods were compared by Passing-Bablok regression and Bland-Altman plot by statistical software MedCalc Statistical Software version 17.2 (MedCalc Software, Mariakerke, Belgium).

The performance of the assay was evaluated by use of patient samples, and the results are shown in the Passing-Bablok regression plot [slope with 95% confidence interval (CI), intercept with 95% CI] (Fig. 2). Regression analysis (regression equation $y = 0.467 + 0.987x$; r = 0.980) showed an intercept of 0.4667 (95% CI, −6.0 to 6.3256) and a slope of 0.9867 (95% CI, 0.9302–1.0375). There was not a significant deviation from linearity ($P = 0.86$). The correlation coefficient was 0.98 with a 95% CI from 0.970 to 0.986 with $P < 0.0001$.

The Bland-Altman plot (bias with 95% CI, 95% limits of agreement) (insert in Fig. 2) showed a very good correlation with the HPLC reference method having a nonsignificant bias (2.09%; 95% CI, −0.49 to 4.67) with 95% of the LC-MS/MS–HPLC fluorescent assay differences ranging from −23.86% to 28.04%.

Evaluation of Clinical Samples

For clinical validation, the new method was applied to 95 patient's plasma samples, all assuming other AED as co-therapy (described in Table 1).

Plasma perampanel concentrations, estimated with the D'Agostino–Pearson test, showed a normal distribution ($P > 0.05$). The presence of outliers data was excluded by the Grubbs test. No significant differences were noted with regard to age and gender. Perampanel plasma concentrations in patients' samples at steady state, determined with the LC-MS/MS method, ranged from 35.0 to 985.0 ng/mL in a dose range 2–12 mg (mean 231.4 ng/mL, median 184.0 ng/mL) (data are shown in Table 1) and are in line with previously reported data.[6–8]

Patients comedicated (n = 17) with AEDs known as neutral (no enzyme inducer or inhibitor) (LTG, LEV, ZNS, CLB, CLZ) had a mean perampanel plasma concentration of 313.2 ± 232.4 ng/mL. Patients comedicated with enzyme inducers (CBZ, PHT, PB, PRM, OXCB, and TPM) (n = 49) had a lower mean perampanel concentration of 163.2 ± 85.2 ng/mL. The mean perampanel concentration of patients treated with VPA (n = 15) as concomitant therapy (enzyme inhibitor) was 359.3 ± 199.3 ng/mL. Patients comedicated with known enzyme inducers plus VPA (n = 14) had an intermediate mean plasma concentration of 233.5 ± 132.7 ng/mL (Fig. 3).

DISCUSSION

HPLC and LC-MS/MS protocols for measuring perampanel concentrations in plasma or serum have been reported.[9,14–19] These protocols are based either on liquid–liquid extraction or protein precipitation. Our method is based on a simple protein extraction with less sample volume than those reported (50 μL instead of 100 μL). Compared to previously published methods for perampanel monitoring, our method is the first that allows simultaneous determination of 18 different AEDs; most of them are often present in patient's

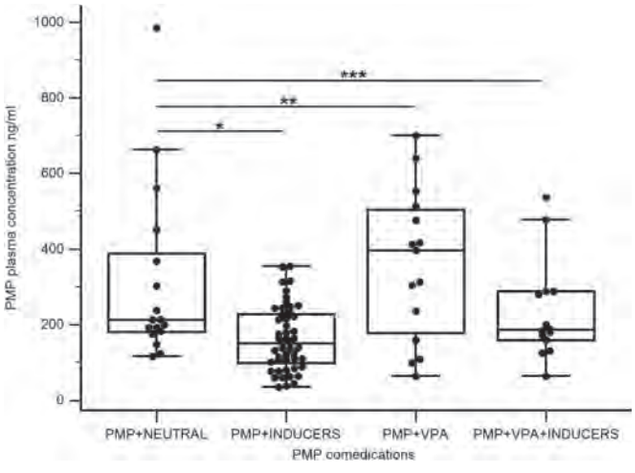

FIGURE 3. Patient's perampanel (PMP) plasma concentrations (ng/mL) according to concomitant therapies (described in text and Table 1). The median of PMP plasma concentrations of the PMP + Neutral group was significantly different from that of PMP + Inducers (*$P = 0.009$), but not significantly different from that of PMP + VPA (**$P = 0.168$) and PMP + VPA + Inducers (***$P = 0.583$).

samples as comedication. The method's linearity range is adequate for perampanel monitoring in clinical settings if we consider that suggested reference values of plasma concentration vary from 180 to 980 ng/mL.[8] Our method showed to be linear in a broader range of concentrations (from 2.5 to 2800 ng/mL). The linearity ranges reported by Mano et al[16] extend between 0.25 and a maximum of 500 ng/mL, whereas Patsalos et al[8] reported an assay range of 2–800 ng/mL. Thus, our methods cover a more extended linearity range. The reported LLOQ for the previously published methods are lower; however, our LLOQ is sufficient for the measurement of clinically relevant drug concentrations. Recently, a similarly fast and simple method for perampanel extraction from clinical samples has been published by Mohamed S. et al[19] coupled with an HPLC fluorescent assay and limited to perampanel monitoring. Calibration curve in the Mohamed et al method ranged from 20 to 1000 ng/mL. Our data confirm previous studies about dose and concentration ratios, and no effect from variables such as gender and age.[7,8] The segregation of patients in the different groups of concurrent therapies reflects published data[6–8,14] with significant differences in perampanel plasma concentration between the group of patients receiving cotherapy with neutral AEDs (mean concentration 313.2 ± 232.4 ng/mL) and those with enzyme inducer AEDs (163.2 ± 85.2 ng/mL) with a significant difference in mean concentration of −48% ($P = 0.009$). Patients comedicated with VPA (a well-known enzyme inhibitor) conversely showed a higher mean concentration of 359.3 ± 199.3 ng/mL. The percentage difference in mean concentration between these 2 groups is of 15% and is not sufficient to achieve statistical significance ($P = 0.168$). These data are concordant with data published recently from Mohamed et al and Patsalos et al[8,19] If we consider that patients comedicated with VPA and inducing AEDs showed an intermediate mean concentration (233.5 ± 132.7 ng/mL) with respect to those comedicated with neutral AEDs (313.2 ± 232.4 ng/mL) ($P = 0.583$), we must assume that metabolic pathways driving perampanel and VPA interaction remain to be further elucidated. Moreover, we have to consider the low number of patients comedicated with VPA in these studies: 14/95 in this study, 9/30 in the Mohamed et al study,[19] and 25/107 in the study from Patsalos et al[8] These observations about a possible pharmacological interaction between these 2 drugs need to be confirmed with further studies in a larger cohort of VPA comedicated patients, that is now undergoing in our hospital.

All the studies we considered so far are focused on perampanel plasma concentration monitoring. Our method is the first to introduce simultaneous monitoring of perampanel and concomitant AEDs with no analytical interferences. All analytes are detectable after a simple protein precipitation, in a single run, in a limited time of 10 minutes and assay ranges are well encompassing the therapeutic ranges defined in the recently released "Consensus Guidelines for therapeutic drug monitoring in neuropsychopharmacology: update 2017"[20] and shown in Table 2. Moreover, the extraction recovery was above the limits, whereas imprecision and inaccuracy were within accepted limits indicated by the most recent version of guidelines on bioanalytical method validation released by EMA.[12] This possibility may be of interest to the laboratories monitoring perampanel and other AEDs as perampanel is only approved as adjunctive therapy.

CONCLUSIONS

The LC-MS/MS method we developed for quantification of perampanel in human samples shows sensitivity, precision, linearity, and accuracy within the acceptance criteria. The method can be easily applied in laboratory settings, and furthermore, the new method is suitable to monitor perampanel plasma concentration in all those patient's samples containing a number of AEDs, allowing simultaneous determination of concomitant drugs with no interferences.

Our data on patient's sample well correlate with biological findings on perampanel kinetics, so our method is suitable for routine application in TDM.

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LC/MS

FORENSIC TOXICOLOGY

POSTER

2° Congresso Nazionale Sipmel Montesilvano (PE)
4-5-6 Ottobre 2016

"NUOVO METODO DI SCREENING TOSSICOLOGICO RAPIDO IN CROMATOGRAFIA LIQUIDA/SPETTROMETRIA DI MASSA (LC/MS)"

NUOVO METODO DI SCREENING TOSSICOLOGICO RAPIDO IN CROMATOGRAFIA LIQUIDA/SPETTROMETRIA DI MASSA (LC/MS)

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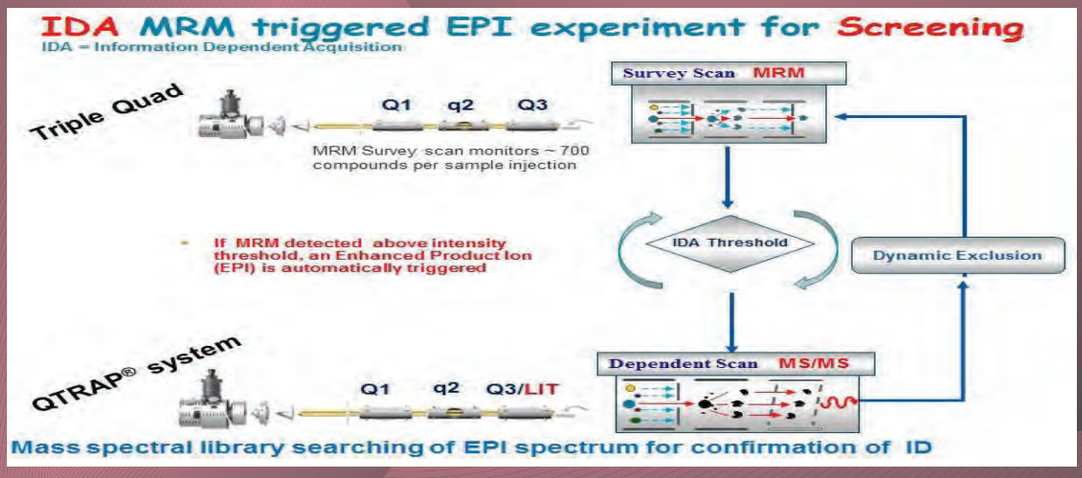
Il metodo analitico da noi sviluppato ha lo scopo di ampliare i composti determinabili per le indagini tossicologiche nelle diverse matrici biologiche, permettendo in un'unica corsa cromatografica in detector di massa l'analisi qualitativa di centinaia di composti in modalità positiva e negativa, nel plasma, nel sangue e nelle urine.
Il settaggio strumentale prevede l'utilizzo di una standard mix con cui si configurano i tempi di ritenzione, gli ioni primari e gli ioni di frammentazione di molecole di riferimento. La preparazione dei campioni prevede o una semplice diluizione, o una deproteinizzazione con utilizzo di standard interni. La corsa cromatografica ha una durata complessiva di 18 minuti, il metodo prevede l'utilizzo di una colonna Restek 5μm PFP propyl (60 Å, 50 x 2.1 mm).
La determinazione dei composti avviene tramite il criterio 'Information Dependant Acquisition' (IDA concept). Attraverso l'utilizzo di questo criterio è possibile stabilire un'intensità minima che il segnale deve raggiungere affinché venga analizzato in MS/MS (Massa/Massa). Quindi solo nel momento in cui viene soddisfatta la condizione di intensità, il software provvede autonomamente ad innescare l'Enhanced Product Ion (EPI) delle molecole presenti, cioè la frammentazione delle stesse tramite una scansione MRM (Multiple Reaction Monitoring) e contemporaneamente effettua una esclusione dinamica dei composti che non soddisfano il criterio di intensità stabilito (IDA filters).



Le molecole cosi determinate possono essere confrontate con quelle presenti nella libreria ottenendo oltre all'identificazione, il grado di purezza e il grado di concordanza. L'utilizzo del nuovo metodo ha avuto modo di trovare applicazione nella rilevazione di 702 sostanze tra cui farmaci, droghe d'abuso, agenti dopanti e metaboliti. Il metodo di screening LC/MS è stato testato mediante l'analisi di 52 campioni di urina e 47 campioni ematici risultati positivi per sostanze d'abuso, tutte le positività sono state confermate utilizzando metodi specifici quantitativi in LC/MS.
I tempi di esecuzione per l'analisi in LC/MS si sono rilevati assai ridotti se equiparati a dosaggi immunoenzimatici, consentendo inoltre recuperi vicini al 100% degli analiti e garantendo allo stesso tempo livelli superiori di sensibilità e specificità analitiche. Il metodo sviluppato in questo lavoro si è rilevato perfettamente adeguato sia per un'indagine di screening preliminare anche a carattere di urgenza, sia per essere utilizzato quale indagine di conferma del dato ottenuto attraverso le più comuni e limitate determinazioni in immunoenzimatica.
La possibilità di poter configurare il metodo a seconda delle esigenze dei singoli laboratori tossicologici attraverso le impostazioni di sensibilità e l'utilizzo di una libreria di molecole determinabili in continuo aggiornamento, fanno della nuova metodica uno strumento economico e versatile per un rapido screening tossicologico applicabile alla diagnostica di laboratorio.

2°CONGRESSO NAZIONALE SIPMEL MONTESILVANO (PE), 4-5-6 OTTOBRE 2016

POSTER

66TH ASMS Conference on Mass Spectrometry and Allied Topics
June 3 - 7, 2018,
San Diego, California

"FULLY AUTOMATED ANALYSIS OF THC AND METABOLITES IN WHOLE BLOOD"

SHIMADZU

Fully automated analysis of THC and metabolites in whole blood

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Overview

- A method was developed to measure THC and its metabolites, THC-COOH and 11-OH-THC from whole blood.
- Samples were analyzed using fully automated sample preparation with on-line LC-MS/MS with no need for human intervention after sample loading.
- The Shim-pack MAYI-ODS trap column, which contains particles with outer an surface of silica gel (50 µm), are coated with a hydrophilic polymer so only interior pores are chemically modified by octadecyl radicals (ODS).
- Proteins and other macromolecules were prevented from entering the pores and quickly eluted to waste before switching the valve position to the analytical column for separation and elution of retained compounds.

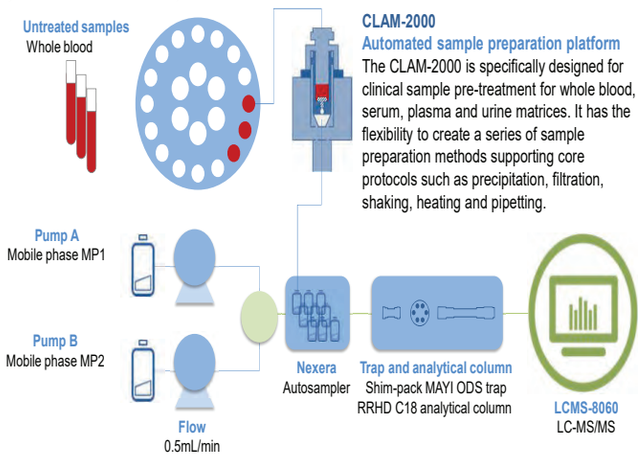
1. Introduction

Tetrahydrocannabinol (THC) is the most commonly encountered cannabinoid and is rapidly metabolized to THC-COOH and 11-OH-THC. Although used in medicinal applications it is commonly used as drug of abuse because of its psychoactive properties. Typically the quantification of drugs of abuse in blood is performed is performed by immunoassay however these assays can lead to false-positive results. For this reason more accurate and precise methods have been developed using LC-MS/MS. In this work we report a fully automated method that performed on-line sample preparation and analysis in whole blood samples using a novel trap column enabling high throughput analysis.

2. Methods

CLAM-2000+Drugs of Abuse Reagent Kit

The automated sample preparation method required 150 µL of extraction buffer containing internal standard added to a PTFE filter vial (0.45 µm pore size, pre-conditioned with 30 µL methanol). 50 µL of whole blood was added. The mixture was shaken for 60 seconds (2000 rpm) then filtered (by application of vacuum pressure -60 to -65 kPa) for 60 seconds into a collection vial. Finally, 50 µL of the extract was injected into the LC-MS/MS system.



LC-MS/MS analysis

Sample analysis was performed using a trap & analytical column configuration. All data was acquired by multiple reaction monitoring (MRM) with 2 transitions measured per compound.

Figure 1: Automated sample preparation for clinical sample analysis fully integrated with LC-MS/MS detection. Mobile phase and sample preparation reagents (including internal standard) were provided by Euraka Drugs of Abuse kit P/N 89010: http://www.eurekaone.com)

2-1. LC-MS/MS analysis of THC metabolites

A drugs of abuse reagent kit was used in the automatic sample preparation platform for THC and its metabolites in whole blood. To deliver a robust, routine method a Shim-pack MAYI-ODS trap column was used to reduce matrix effects, ion suppression and increase analytical performance

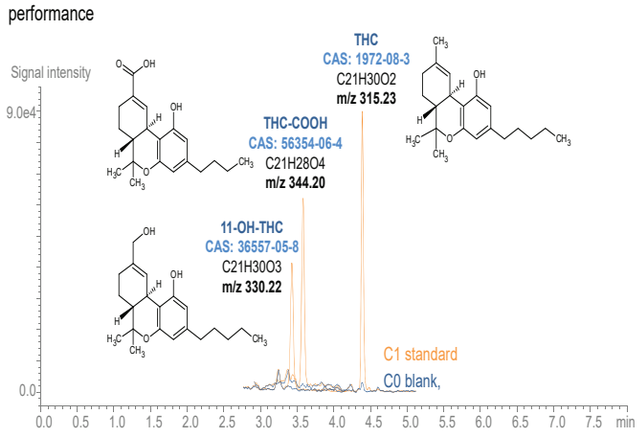


Figure 2. MRM chromatograms for THC and its primary metabolites 11-OH-THC and THC-COOH following a whole blood sample extraction.

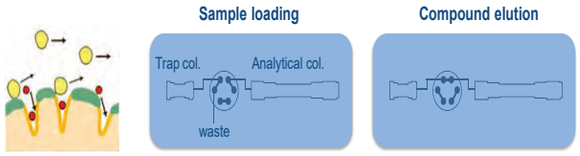


Figure 3. The Shim-pack MAYI-ODS trap column was used with a binary gradient system. It contains particles with outer an surface of silica gel (50 µm) that are coated with a hydrophilic polymer. Proteins and other macromolecules are prevented from entering the pores and quickly eluted to waste before switching the valve position to the analytical column for separation and elution of retained compounds.

Table 1. Analytical conditions for the LC-MS/MS system.

Liquid chromatography			Mass spectrometry	
UHPLC	NexeraX2 LC system		LC-MS/MS	LCMS-8060
Trap column	Shim-pack MAYI ODS (5 x 2mm)		MRM Dwell time	13-24 msec [compound dependent]
Analytical column	RRHD C18 (50 x 2.1, 1.8um)		Pause time	1 msec
Flow rate	0.5 mL/min		Interface/Block/Desolvation	300°C/400°C/250°C
Binary Gradient	Time (mins)	%B	Heating/Drying gas	10 L/min
	0.30	5	Nebulising gas	3 L/min
	0.35	60	CID gas pressure	250kPa
	5.10	100	Interface voltage	1.5 kV
	8.00	100	Ionisation mode	Heated ESI
	8.01	5		
	10.00	Stop		

Table 2. MRM's monitored for THC analysis in whole blood by LC-MS/MS

Compound	Type	RT (min)	Quan. m/z	CE (V)	Ref. m/z	CE (V)	Calibration range (ppb)
THC	Target	4.421	315.25 > 193.15	-24.0	315.25 > 259.15	-18.0	1.5 - 12.0
ISTD THC D3	ISTD	4.419	318.30 > 196.20	-22.0	318.30 > 123.10	-33.0	
11-OH-THC	Target	3.465	331.25 > 193.10	-25.0	331.25 > 200.95	-23.0	0.5 - 10.0
ISTD 11-OH THC D3	ISTD	3.459	334.30 > 196.00	-25.0	334.30 > 175.20	-23.0	
THC-COOH	Target	3.614	345.20 > 299.10	-20.0	345.20 > 192.95	-27.0	1.0 - 8.5
ISTD THC-COOH D9	ISTD	3.595	354.30 > 308.30	-20.0	354.30 > 196.10	-29.0	

3-1. Quantitation

The automated sample preparation method, using a specific reagent kit for drugs of abuse has been validated for the detection and quantification of the cannabinoids -Δ9-tetrahydrocannabinol (THC) and its two major metabolites 11-Hydroxy-Δ9-tetrahydrocannabinol (11-OH-THC) and 11-nor-9-carboxy-delta-9-tetrahydrocannabinol (THC-COOH) using a whole blood sample volume of 50 uL. Stable isotope labelled internal standards were used for each cannabinoid. Several calibrators were analysed with each batch and used to assess the linearity of response. Figure 4 shows sample calibration curves derived from the method. Calibrations were linear over the concentration range studied for each analyte. The data indicates that the method meets the criteria required for routine analysis of cannabinoids in whole blood samples.

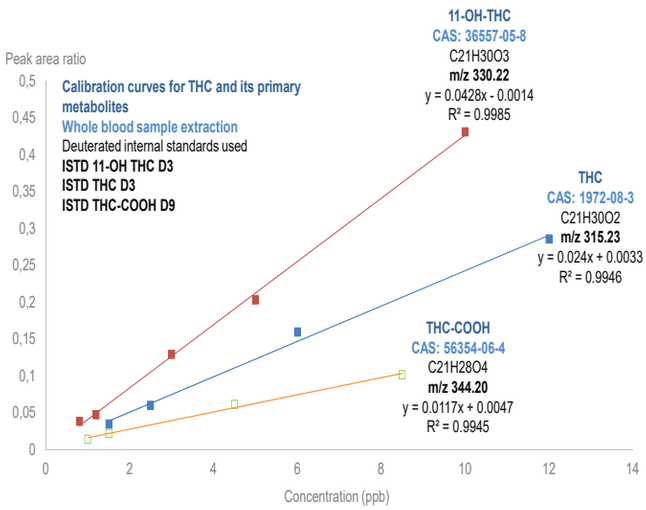


Figure 4 Calibration curves for THC and its primary metabolites 11-OH-THC and THC-COOH following an automated whole blood sample extraction and LC-MS/MS detection. Regression coefficients (R^2) are typically greater than 0.994.

Compound	Concentration (ppb) Set	Measured	% Deviation	% Accuracy
THC	1.50	1.44	-3.88	96.1
	2.50	2.47	-1.36	98.6
	6.00	6.48	7.97	108.0
	12.00	11.57	-3.55	96.5
11-OH-THC	0.80	0.89	11.72	111.7
	1.20	1.11	-7.93	92.1
	3.00	3.04	1.34	101.3
	5.00	4.78	-4.32	95.7
	10.00	10.18	1.75	101.8
THC-COOH	1.00	1.03	3.25	103.3
	1.50	1.58	5.19	105.2
	4.50	4.41	-2.02	98.0
	8.50	7.30	-14.14	85.9

Table 3 Using the reagent kit calibrators and automated sample extraction method with LC-MS/MS detection, the accuracy of the method for all levels and all compounds was within 85-115%.

Figure 4. Calibration curve results from THC and its metabolites, THC-COOH and 11-OH-THC from whole blood. All compounds achieved a regression coeficient R^2 > 0.99 and accuracy 85-115%. Blank calibration samples demonstrated very low noise demonstrating capacity to measure lower levels or lower injection volumes to extend the calibration curve to higher blood concentrations.

3-2. Quality control analysis

THC	Peak Area QC-1	Peak Area QC-2
Sample A	314890	3019237
Sample B	311908	2836560
%RDS	0.67	4.41

11-OH-THC	Peak Area QC-1	Peak Area QC-2
Sample A	91476	914460
Sample B	91009	1052285
%RDS	0.36	9.91

THC-COOH	Peak Area QC-1	Peak Area QC-2
Sample A	172659	1938428
Sample B	185918	2007076
%RDS	5.23	2.46

Table 4 Inter-day quality control samples were analyzed in duplicate and demonstrated acceptable precision (better than 15% RSD).

Figure 5: The CLAM-2000/LCMS-TQ platform is configured to synchronise sample preparation during sample analysis of the previous injection resulting in a true high throughput system.

4. Conclusions

- This work presents the first fully automated sample preparation and analysis of THC and its primary metabolites in whole blood samples for routine screening laboratories. The method uses an automated samples preparation platform and an integrated trap column to increase LC-MS/MS performance.
- The CLAM-2000 supports integrated calibrators and quality controls throughout the batch analysis, parallel analysis and sample preparation and can be fully adapted to a range of sample preparation protocols including reagent aliquoting, ISTD addition and extraction for automated LC-MS/MS analysis.
- A reagent kit was used to quantitate THC and its primary metabolites.
- The method delivered linear calibration curves over the concentration range studied.
- CV% values complied with the CLSI reference intervals. The accuracy of the method was found to be between 85% and 115%.

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