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Determination of the $GABA_B$ receptor agonist CGP 44532 (3-amino-2-hydroxypropylmethylphosphinic acid) in rat plasma after pre-column derivatization by micro-high-performance liquid chromatography combined with negative electrospray tandem mass spectrometry

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Abstract

An assay, based on pre-column derivatization and micro-high-performance liquid chromatography–tandem mass spectrometry, was developed for the determination of the $GABA_B$ agonist CGP 44532 in rat plasma. CGP 44532, a highly polar 3-amino-2(*S*)-hydroxypropylmethylphosphinic acid, presented difficulties in developing a chromatographic method for the analysis of the compound in rat plasma. Instead of analyzing the target compound directly, it was derivatized prior to separation to a 4-nitrobenzylcarbamate isopropyliden derivative. In order to reach the required quantitation limit, on-line solid-phase extraction was utilized for sample clean-up and reversed-phase micro-column high-performance liquid chromatography, for separation of the plasma samples. The separated compounds were detected by negative electrospray tandem mass spectrometry in selected reaction monitoring mode. The derivatives show good chromatographic and mass spectrometric properties and both the target compound and the internal standard, could be eluted as symmetrical peaks with good signal/noise ratio. The MS–MS detection was selective and sensitive due to the straight fragmentation pattern. After injection of 200-μl sample aliquots, the limit of quantification was 10 ng ml^{-1}. The analytical assay is useable in the range of 10–500 ng ml^{-1}.

Keywords: 3-Amino-2-hydroxypropylmethylphosphininc acid; Nitrobenzylcarbamate isopropylidene derivative

1. Introduction

γ-Aminobutyric acid (GABA, **I**) is the major inhibitory neurotransmitter in the central nervous system [1]. It interacts with two types of receptors designated $GABA_A$ and $GABA_B$ [2]. $GABA_B$ predominates in the molecular layer of cerebellum, the thalamus and the dorsal horns of the spinal cord [3]. A major function of $GABA_B$ receptors is the modulation of the release of several neurotransmitters, such as glutamate [4], dopamine, noradrenaline, serotonin [5], etc., via pre-synaptic $GABA_B$ binding sites.

GABA plays an important role in the spinal cord,

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where it is involved in the regulation of reflex activity responsible for maintaining normal muscle tone [6,7]. The $GABA_B$ receptor agonist D,L-baclofen (Lioresal, **II**) a lipophilic GABA derivative [8], acts at the spinal cord level primarily by reducing spinal reflex disinhibition. In most neurological disorders, e.g., multiple sclerosis, spinal cord injury, cerebral palsy, spasticity and neural pain baclofen is the first choice of therapy [9,10].

A new series of selective, more potent, and better tolerated $GABA_B$ receptor agonists was discovered by replacing the carboxylic acid group of GABA, GABOB (i.e., γ-amino-β-hydroxybutyric acid, **III**) or baclofen, respectively (see Fig. 1), by various phosphinic acid residues (e.g., Ref. [11]). The most prominent candidate was 3-amino-2(*S*)-hydroxypropylmethylphosphinic acid (CGP 44532, **IV**) for which a large collection of pre-clinical data indicates a significant superior to D,L-baclofen.

In this paper, we describe the method development approach and the optimization of conditions to measure the highly polar CGP 44532 and its ethyl homologue 3-amino-2-hydroxypropylethylphosphinic acid (CGP 47399A, internal standard). The analytical method which employed pre-chromatographic derivatization, column-switching and reversed-phase micro-high-performance liquid chromatography in combination with negative electrospray tandem mass spectrometry (μ-HPLC–ES-MS–MS), allowed quantification of CGP 44532 in plasma in the concentration range of 10 to 500 ng ml^{-1}.

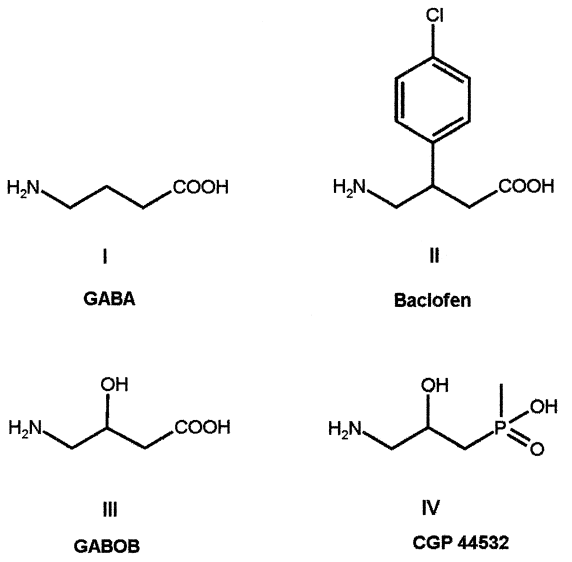


Fig. 1. Chemical structures of GABA, Baclofen, GABOB and CGP 44532.

2. Experimental

2.1. Chemicals and supplies

Samples of CGP 44532 and CGP 47399 (internal standard) (Table 1) were obtained from Novartis Pharma, Pharmaceutical Research (Basel, Switzerland).

Table 1
Structures and formulas of the compounds investigated

Compound code	R	Formula	M_r
CGP 44532 (*S*)	CH_3	$C_4H_{12}NO_3P$	153
CGP 47399 (*rac*)	C_2H_5	$C_5H_{14}NO_3P$	167

2.2. Sample preparation

2.2.1. Plasma ultrafiltration

About 300 μl blank rat plasma samples or plasma samples obtained from an early ADME study (adsorption–distribution–metabolism–excretion) in rat were transferred to disposable ultrafiltration tubes (Amicon Centrifree No. 4104) containing a regenerated cellulose membrane with a molecular mass cut-off of 30 000. For quantitative measurements, 75 ng of 3-amino-2-hydroxypropylethylphosphinic acid was added as internal standard (150 μl of a stock solution containing 50 μg CGP 47399 per 100 ml). The tubes were centrifuged for 30 min at 1600 *g* and 37°C. About 100 μl of filtrate was obtained after centrifugation. Aliquots of the resulting plasma–water samples were derivatized and analyzed.

To assess the unspecific binding of the test compound onto the filtration equipment, ^{14}C-labeled 3-amino-2-hydroxypropylmethylphosphinic acid was dissolved in water and submitted to ultrafiltration as described above. Radioactivity was measured before and after filtration. No difference was observed.

2.2.2. Derivatization reaction

The general reaction scheme of the derivatization of 3-amino-2-hydroxypropylalkylphosphinic acids in aqueous solution is shown in Fig. 2. In one step CGP 44532 and CGP 47399 were converted with acetone and 4-nitrobenzylchloroformate, under basic conditions, to the final isopropylidene-carbamate derivatives. The related mass spectra of the derivatized parent compound and internal standard are shown in Figs. 4 and 5.

2.2.3. Preparation of the plasma samples

A 50-μl volume of ultrafiltrated (blank) rat plasma containing 12.5 ng internal standard/50 μl plasma (corresponding to 250 ng ml^{-1}) was spiked with 0.5, 1, 2.5, 5, 12.5, or 25 ng CGP 44532, respectively, (corresponding to 10, 20, 50, 100, 250 or 500 ng ml^{-1}) and mixed with 400 μl acetone and 50 μl borate buffer, pH 9 (6.56 g $Na_2B_4O_7 \cdot 10H_2O$+0.93 g KH_2PO_4/100 ml water).

The mixture was vortex-mixed for 20 s. After shaking at room temperature for 45 min, 100 μl of a 4-nitrobenzylchloroformate solution (50 mg in 1 ml acetone) was added and shaken for 30 min.

After completion of the derivatization reaction the sample volume was reduced, under vacuum (Speed Vac), to a volume of about 100 μl and filled up with 350 μl water–methanol–phosphate buffer, pH 7.5 (5.0 g $Na_2HPO_4 \cdot 2H_2O$+0.66 g KH_2PO_4/100 ml water, 60:30:10, v/v) and 100 μl toluene. Then, this mixture was shaken and centrifuged (5 min at 10 000 rpm).

The plasma samples obtained from the early ADME study were spiked with 12.5 ng internal standard and treated in the same manner as above.

Aliquots of 200 μl of the clear aqueous phases were used for the subsequent μ-HPLC–MS–MS investigation.

2.3. Instrumental

2.3.1. Column-switching set-up and chromatography

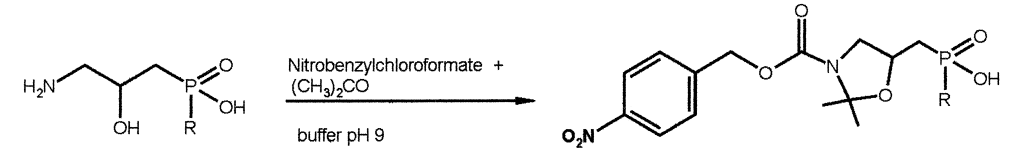
The flow-diagram of the column switching HPLC system and time program of the system controller is shown in Fig. 3.

The system consisted of an automated injection device (HTS PAL, CTC Analytics, Zwingen, Switzerland), a trapping column (Gromsil ODS 5 ST, 20 mm×1 mm), a column-switching system (one electrically-driven microbore six-port switching valve HS 7000E and one low-pressure valve; Lab Source, Reinach, Switzerland) controlled from an Ultra-Plus micro HPLC system (Micro-Tech Scientific, Sunnyvale, CA, USA).

The liquid chromatographic separation was performed using a Phoenix 40 syringe pump (CE Instruments, Milan, Italy) and a Gromsil ODS 5 ST micro-column (150 mm×1 mm, particle size 3 μm). The column temperature was 25°C.

Gradient mobile phase programming was used with a flow-rate of 45 μl min^{-1}. Eluent A was water–acetonitrile (98:2)+0.5 m*M* NH_4HCO_3 100 ml^{-1}. Eluent B was acetonitrile/water (90:10)+0.5 m*M* NH_4HCO_3 100 ml^{-1} (corresponding to E1). A linear gradient was run from 15 to 75% B over 13 min.

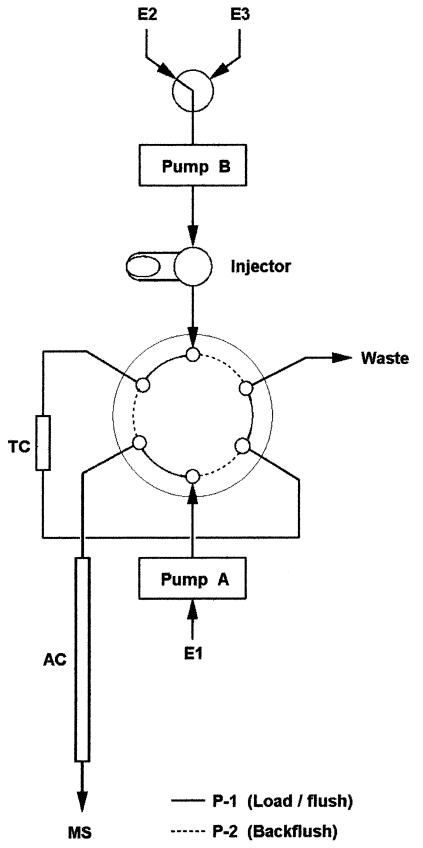
After injection of 200 μl derivatized plasma sample into the sampling loop, the sample was transferred with mobile phase E2 [E2=water–methanol (96:4)+0.5 m*M* NH_4HCO_3 100 ml^{-1}] to the trapping column at a flow-rate of 100 μl min^{-1} for 2



R = CH_3 (CGP 44532)

R = C_2H_5 (CGP 47399)

Fig. 2. Reaction pathway of the derivatization of 3-amino-2-hydroxypropylalkylphosphinic acids in aqueous solution.



Columns

Trapping column (TC) : Gromsil ODS-5 ST, 5µm, 20 x 1 mm

Analytical column (AC): Gromsil ODS-5 ST, 3µm, 150 x 1 mm

Events

Load : Transfer sample volume to TC with eluent E2

Desorbtion : TC and AC connected. Flush of trapped components to AC with eluent E1

Flush : Flushing of TC with eluent E3 to remove endogenous components

Eluents :

E1 : Gradient:
Eluent A = Water/acetonitrile (98+2) + 0.5 mM NH_4HCO_3/100 ml
Eluent B = Water/acetonitrile (10+90) + 0.5 mM NH_4HCO_3/100 ml

E2 : Isocratic:
Water/methanol (96 + 4) + 0.5 mM NH_4HCO_3/100 ml

E3 : Isocratic:
Water/acetonitrile/acetone (5/60/35)

Program for controlling the column switching system

Position of switching valve	P-1		P-2	P-1				
Process TC	Load		Desorb	Flush			Equilibration	
Eluent of TC	E2		E1	E3	E2			
Flow rate pump B (µl/min)	100	250					100	
Process AC	Idling		Separation				Equilibration	
Eluent of AC (% E1- B)	5		15			75	100	5
Flow rate pump A (µl/min)	45							
Time (min)	0	3	6	12	16	19	20	25

Fig. 3. Flow diagram, set-up and time sequence of the column-switching µ-HPLC system.

min. After flushing the trapping column for 3 min with 250 μl E2, the switching valve was turned to the alternate position, so that the trapping and analytical columns were in-line. Mobile phase E1 transferred the enriched analytes through the trapping column onto the analytical column. After 10 min transfer time, the switching valve was turned back to the starting position in order to disconnect the trapping column from the analytical column. In this position the trapping column was flushed with E3 [E3=water/acetonitrile/acetone (5:60:35)] at a flow-rate of 250 μl min^{-1} for 4 min, in order to remove retained endogenous compounds. After washing, the trap was re-equilibrated at a flow-rate of 100 μl min^{-1} with mobile phase E2 until the next injection was made.

2.3.2. Mass spectrometry

The column eluent was introduced directly into the ion source of a Bio-Q triple quadrupole mass spectrometer (Micromass, Manchester, UK). The ionization technique employed was negative electrospray (ES). The sprayer voltage was kept at 3400 V. The cone voltage of the ion source was kept at a potential of 40 V.

The mass spectrometer was used in the MS–MS mode, utilizing collision induced dissociation in Q2 (collision chamber). Collision gas was argon. The collision energy was set at 25 V. The compounds, were detected by selected reaction monitoring (SRM).

The reactions monitored were m/z 371→m/z 152 (CGP 44532, derivatized 3-amino-2-hydroxypropylmethylphosphinic acid) and m/z 385→m/z 152 (internal standard, derivatized 3-amino-2-hydroxypropylethylphosphinic acid), respectively (see Figs. 4 and 5). Dwell time was 0.2 s. Representative mass chromatograms of calibration samples containing 100 ng and 500 ng CGP 44532, respectively, are shown in Figs. 6 and 7.

2.3.3. Method validation

For validation, pooled ultrafiltrated blank rat plasma was used. The validation samples covered the concentration range between 10 to 500 ng ml^{-1}. The validation based on guidelines established during the conference on method validation for quantitation of drugs in biological media [12]. According to these guidelines the intra-day precision was determined at three concentration levels (10, 100 and 500 ng ml^{-1}). The inter-day precision was studied on 3 different days at the same concentrations. At concentrations around the limit of quantification (10 ng ml^{-1}) as well as at higher concentrations the vari-

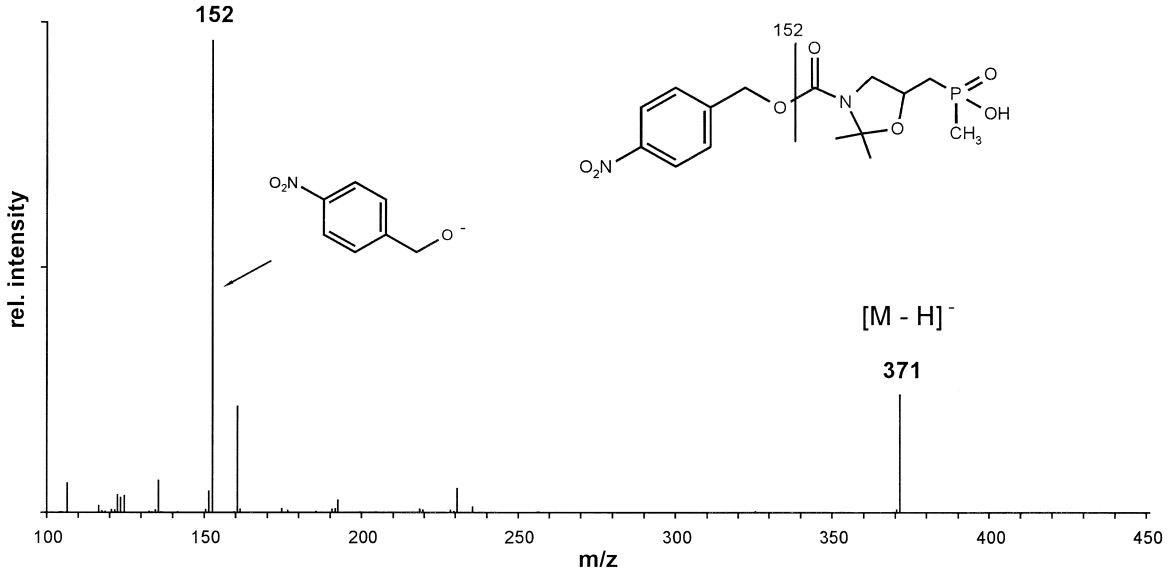


Fig. 4. Negative electrospray daughter-ion mass spectrum of derivatized CGP 44532.

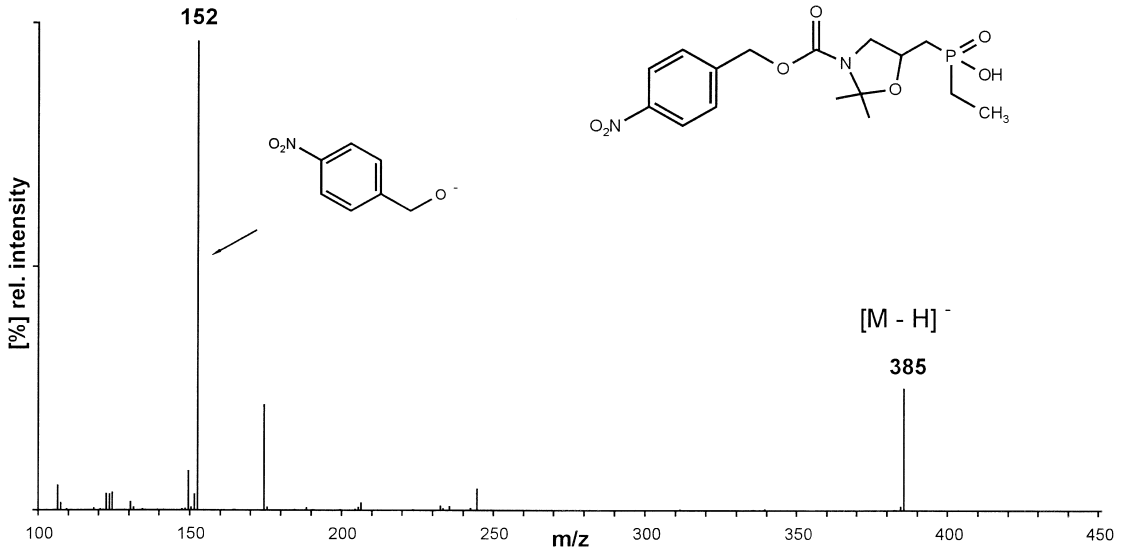


Fig. 5. Negative electrospray daughter-ion mass spectrum of derivatized CGP 47399 (internal standard).

ability of precision and accuracy should not exceed 15% of relative standard deviation (RSD). The respective data are summarized in Table 2.

The usual estimation of recovery could not be realized due to the lack of both, pure derivatized parent compound and internal standard. Therefore, the calibration curve was prepared in ultrafiltrated blank plasma as well.

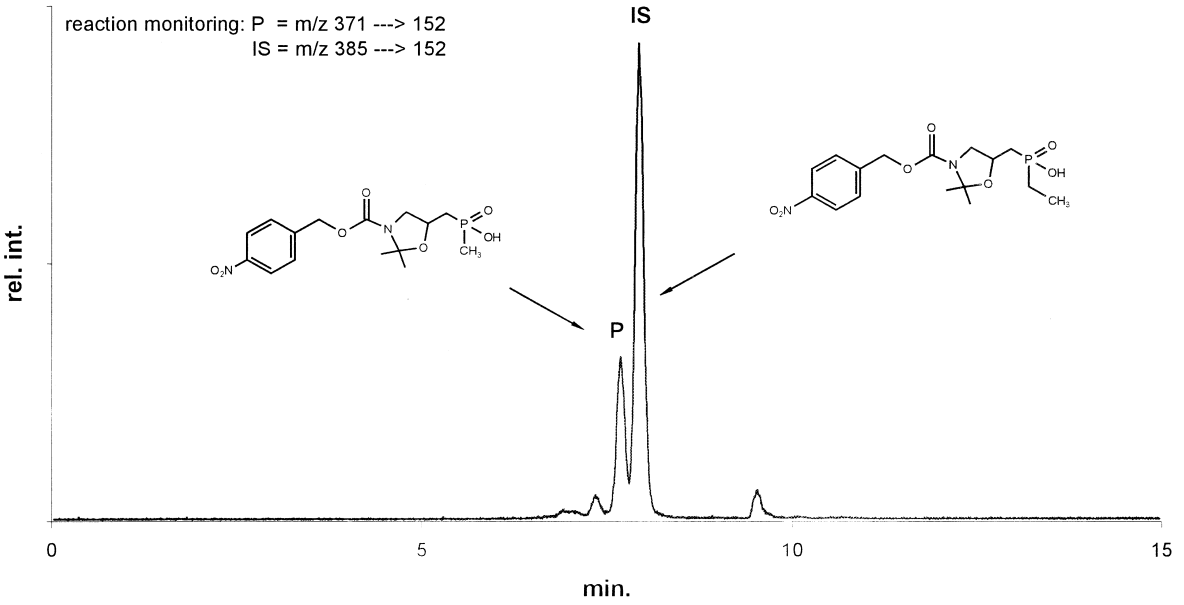


Fig. 6. SRM chromatogram of a plasma–water sample containing 100 ng CGP 44532.

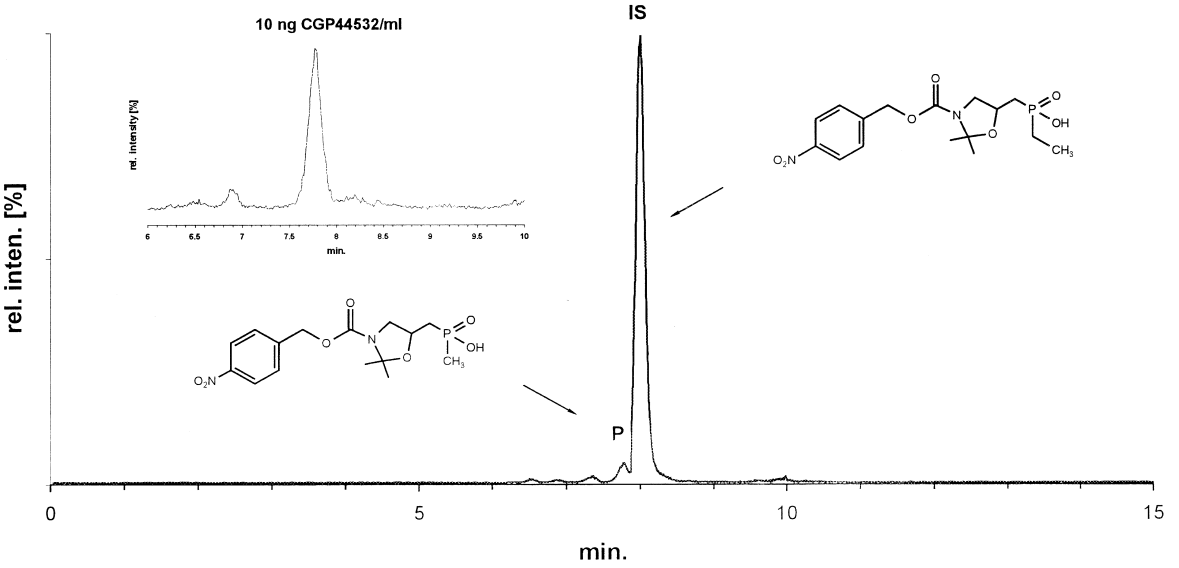


Fig. 7. SRM chromatogram of a plasma–water sample containing 10 ng CGP 44532.

3. Results and discussion

3.1. Mixture analysis of alkylphosphinic acids and related compounds

Analytical assays for the determination of alkylphosphinic acids using gas chromatographic (e.g., Ref. [13]), high-performance liquid chromatographic (e.g., Ref. [14]) or capillary electrophoretic methods (e.g., Ref. [15]) have been reviewed by several authors. However, the analysis of the considerably more polar 3-amino-2-hydroxypropylalkylphosphinic acids, in particular in biological samples such as plasma, urine and various organs, is not covered by these assays and is aggravated by many problems inherent to this compound class.

The general molecular structure and the physico–chemical parameters of CGP 44532 makes it obvious that this class of compounds might be too polar and interactive to be compatible with regular reversed-phase HPLC. Moreover, since the compounds contain no chromophores, UV detection is not possible.

And indeed, the direct reversed-phase HPLC–MS analysis of CGP 44532 on various stationary phases with various mobile phases resulted in extremely short retention values and unacceptably broad peaks. Thus, for a sensitive and efficient analytical assay, measures had to be taken to reduce the polarity of

Table 2
Validation data (accuracy: %; precision: RSD)

CGP 44532	Accuracy (%)				Precision (RSD, %)			
(ng ml^{-1})	Intra-assay			Inter-assay	Intra-assay			Inter-assay
	Day 1 (n=10)	Day 2 (n=7)	Day 3 (n=5)	3 Days (n=22)	Day 1 (n=10)	Day 2 (n=7)	Day 3 (n=5)	3 Days (n=22)
10	116	108	119	114	10	10	4	8
100	95	88	92	92	8	13	13	11
500	98	94	97	96	4	12	9	8

the compound, via a suitable derivative, or to find alternative approaches to HPLC.

A suitable separation method, for charged and highly polar compounds is capillary electrophoresis (CE). Nevertheless, in this particular case it was difficult to achieve the required sensitivity and selectivity in practice, due to the lack of appropriate enrichment methods, or to get reproducible results, due to the unavoidable influence of plasma matrix. Even after ultrafiltration, the serum contains high concentrations of salts and still small amounts of proteins. In CE, the salts cause broadened peaks, while the remaining plasma proteins coated the wall of the separation capillary, thus affecting greatly the reproducibility of the method.

The second alternative approach, solid-phase extraction (SPE) of CGP 44532, for example by anion-exchange SPE, gave good recoveries with aqueous solutions but inconsistent results with ''real'' ultrafiltrated plasma samples (plasma–water). In particular the high concentrations of salts (NaCl content about 140 nmol l^{-1}), reduced the exchange capacity of the used SPE microcolumn systems (Spec 3ML SAX, 15 mg sorbent) significantly. The column capacity was exhausted if the plasma volume exceeded 10 μl. Higher amounts of sorbent, in order to enhance the ion-exchange capacity, lead to irreversible adsorption of the analyte.

The third alternative, is the improvement of the chromatographic properties by reduction of the polarity of CGP 44532 via derivatization. Although, in the case of the 3-amino-2-hydroxypropylalkylphosphinic acid many derivatization methods are conceivable, most assays require apolar reagents, organic solvents and anhydrous conditions. But since the target compound is extremely soluble in water (780 ± 20 g l^{-1}) and nearly insoluble in any organic solvent including dimethyl sulfoxide (DMSO), the necessary pre-concentration prior to derivatization, by established plasma preparation approaches such as liquid–liquid extraction with water-immiscible organic solvents, failed.

3.2. Derivatization in HPLC–MS

Derivatization is a possible way to improve the selectivity of an analytical assay via defined reaction products. Moreover, the subtle use of derivatization can be a strategy for controlling ion intensity and fragmentation in mass spectrometry [16]. Derivatization can be used to increase or decrease fragmentation, to direct fragmentation to new bonds and to enhance the selectivity and sensitivity of the ionization process. The polarity of a molecule can be increased or decreased, and functional groups can be added or removed. The information available from a liquid chromatography–mass spectrometry assay can be vastly improved by inclusion of a derivatization step.

Nevertheless, during the last years, pre-chromatographic derivatization methods, in particular those developed for the analysis of polar compounds by gas chromatography, have lost importance in bioanalytics. New powerful analytical techniques such as immunoassays or HPLC–MS became available, and the fast, specific and sensitive direct determination even of trace amounts of underivatized analytes in biological fluids became the common approach.

However, cases where the analytical problems cannot (or should not) be tackled the direct way, not even with the general purpose separation tool reversed-phase HPLC, pre-chromatographic derivatization is unavoidable. For this purpose, the derivative must be stable against hydrolysis and, if the compound is very polar and if biological fluids have to be analyzed, it is of advantage that the derivatization reaction can be carried out directly in aqueous media.

One class of derivatization reagents, which fulfils all these criteria, in particular for amines or imines, are the so called ''chloroformates''. First reported about 2 decades ago, for the derivatization of amino acids in alkaline aqueous media by Makita et al. [17] they have been developed as general purpose derivatizing agents in GC [18].

Unlike derivatization for GC, where primarily low-molecular-mass reagents such as methyl-ethyl- or isopropylchloroformates are investigated, in order to volatilize the analytes, in HPLC more bulky arylchloroformates, among others as UV or fluorescence detection tags, are preferred [19,20].

3.3. HPLC–MS–MS analysis of derivatized 3-amino-2-hydroxypropylalkylphosphinic acids

The method chosen for the derivatization of 3-amino-2-hydroxypropylalkylphosphinic acids, was

based on the reaction with aryloxycarbonyl chlorides as recently reported by Brückner and Lüpke [19]. The derivatization could be carried out directly in ultrafiltered plasma samples, without any additional clean-up or pre-concentration of the analyte.

However, prior to derivatization it is necessary to remove the bulk of the plasma proteins. The usual deproteinization with organic solvents resulted in very low recoveries, since probably most of the target compound was bounded to the denaturated proteins and could not be extracted. Therefore, for rapid separation of the proteins, the plasma samples were ultrafiltered with a molecular mass cut-off of 30 000.

The derivatization procedure, as described in the Experimental section, was optimized on both the chromatographic and the mass spectrometric properties of the resulting derivative, in order to reach the required limit of quantification (LOQ: 10 ng ml^{-1} plasma) and selectivity. Among the various chloroformates investigated (isobutylchloroformate, 9-fluorenylmethylchloroformate, 4-nitrophenylchloroformate and 4-nitrobenzylchloroformate), the latter reagent diluted in acetone, showed the best results. In aqueous medium, besides the expected formation of the carbamate, in addition a cyclic isopropylidene derivative was formed, between the neighboring amino- and hydroxy-group (see Fig. 2). The formation of the cyclic derivative was unexpected, under the chosen basic conditions (pH 9), but welcome. The mixed derivative was less polar than the expected carbamate and had improved chromatographic and mass spectrometric properties.

Due to the high reactivity of 4-nitrobenzylchloroformate, only a small influence of temperature on the yield was observed. A reaction temperature of 25°C and a reaction time of 30 min were chosen in order to complete the isopropylidene formation.

SPE, which failed for the underivatized compound, came back into the play after derivatization and helps to improve the assay. It protects the analytical column against the excess of reactive 4-nitrobenzylchloroformate and enhances the overall sensitivity by an order of magnitude since larger sample volumes can be injected (up to 200 μl). Although a comparison between off-line SPE and on-line SPE results in a something shorter analysis cycle for the off-line approach, the on-line approach was preferred. The main benefit of on-line SPE was the reduction of laborious clean-up which compensates for the longer cycle times.

Both derivatized 3-amino-2-hydroxypropylalkylphosphinic acids are readily ionized in the chosen mobile phase. Negative electrospray ionization was preferred to positive ionization, since in the negative mode the derivatives could be detected with significantly higher sensitivity and selectivity. The full-scan negative electrospray daughter ion mass spectra of derivatized CGP 44532 and the internal standard (CGP 47399) using the respective $[M-H]^-$ ions at m/z 371 and m/z 385 as parent ions, are shown in Figs. 4 and 5. They undergo extensive, collision induced fragmentation to a dominant daughter ion at m/z 152 (4-nitrobenzylate anion).

Figs. 6 and 7 show representative SRM chromatograms of derivatized CGP 44532 and internal standard (CGP 47399A) in plasma–water, after injection of a 200-μl aliquot of reaction mixture onto the pre-column and subsequent backflushing of the pre-concentrated analytes onto the analytical column. The parent compound and the internal standard could be baseline separated and unequivocally identified. Nevertheless, neighboring peak groups indicate that the mixture situation remains complex and that both, the efficiency of the separation system and the selectivity of the MS–MS reaction channel has to be optimized in order to master the analytical problem.

The calibration range, 10–500 ng ml^{-1}, was selected according to the concentrations expected in the samples. As shown in Fig. 8, the polynomial regression (second order) fitted the data over the entire concentration range. The intercept of the curve did not differ significantly from zero, confirming the good selectivity of the assay. The quantification limit was set at 10 ng ml^{-1}. The precision and accuracy were found to be acceptable. We could meet the required precision criteria of about 15% at the limit of quantification when injecting a 200-μl sample.

3.4. Pharmacokinetic studies

The method was tested, in order to confirm the results of an ADME study in rat plasma. The plasma concentration–time profile after an intravenous (i.v.) dose of 0.5 mg kg^{-1} to rats is shown in Fig. 9. Four hours after i.v. administration, the plasma concentration of GCP 44532 was close or below the LOQ

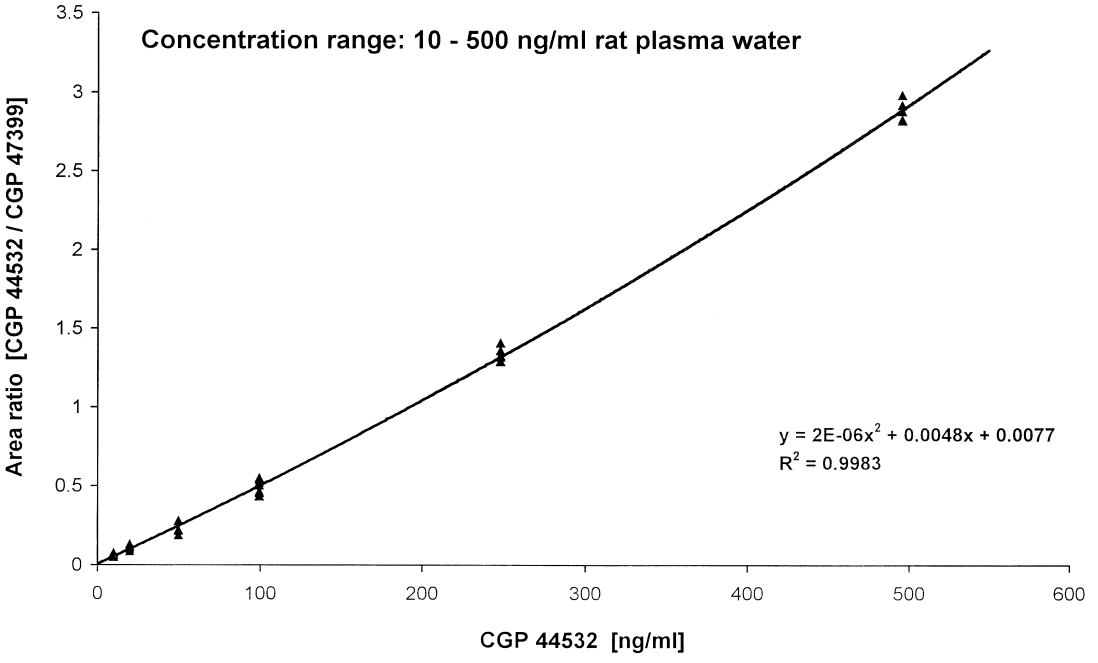
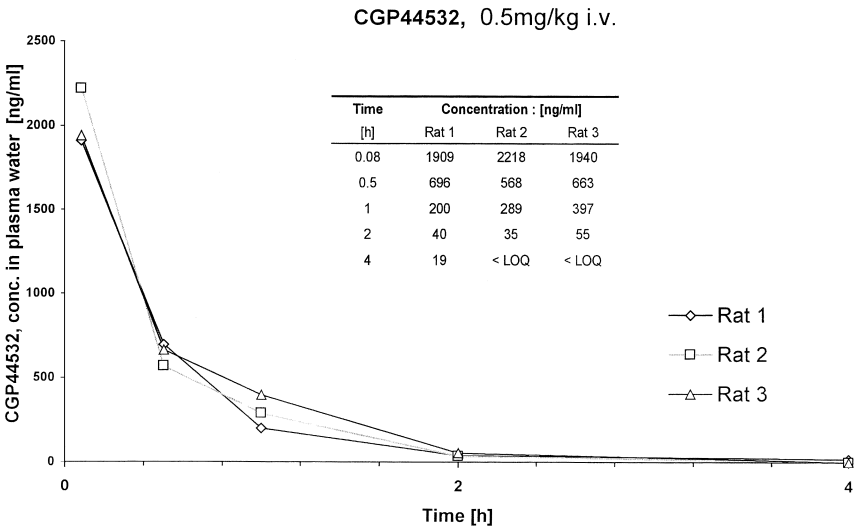


Fig. 8. Calibration for rat plasma samples in the range 10 to 500 ng ml^{-1} single i.v. 0.5 mg kg^{-1} dose of CGP 44532.



Time	Concentration : [ng/ml]		
[h]	Rat 1	Rat 2	Rat 3
0.08	1909	2218	1940
0.5	696	568	663
1	200	289	397
2	40	35	55
4	19	< LOQ	< LOQ

Fig. 9. Plasma concentration–time profile of CGP 44532 in rat plasma following a single i.v. 0.5 mg kg^{-1} dose.

(10 ng ml^{-1}). This rapid elimination corresponds to the results of the ADME study.

4. Conclusion

This work demonstrates that after optimized pre-chromatographic derivatization in aqueous medium, a highly polar compound containing phosphinic acid, primary amino and hydroxy groups (CGP 44532) can be successfully analyzed in rat plasma by on-line SPE in combination with μ-HPLC–MS–MS. Except for the ultrafiltration and derivatization the assay is fully automated and allows unattended operation. The method has proven to be sensitive, selective, accurate and precise in routine application.

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