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Resolution of catecholic tetrahydroisoquinoline enantiomers and the determination of *R*- and *S*-salsolinol in biological samples by gas chromatography–mass spectrometry

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Abstract

Tetrahydroisoquinolines (TIQs) might be formed endogenously and can act centrally to promote a mechanism governing alcohol drinking behaviour. The possibility that biosynthesis occurs through a stereospecific enzymatic reaction is considered. Several TIQs were transformed into diastereomers by a two-step derivatization with N-methyl-N-trimethylsilyltrifluoracetamide and *R*-(−)-2-phenylbutyrylic acid and were analyzed by gas chromatography–mass spectrometry (GC–MS). High resolution of the TIQ enantiomers was achieved. This method was applied to the quantification of the enantiomers of salsolinol (SAL) in urine and plasma of healthy humans. Deuterated SAL was used as the internal standard. SAL was extracted from biological material using phenylboronic phase cartridges and transformed into diastereomers. The sensitivity and specificity of the assay permit the determination of the enantiomeric composition of SAL in plasma and urine. The limit of quantification was found to be 100 pg/ml for each enantiomer. The described method has the advantage that optimal resolution of the SAL enantiomers without peak overlapping between analyte and other compounds can be achieved. Contrary to other findings, our GC–MS studies have demonstrated that endogenously formed SAL is racemic in plasma as well as in urine of healthy subjects.

1. Introduction

Opiate-active tetrahydroisoquinoline alkaloids (TIQs), the Pictet–Spengler condensation products of catecholamines and aldehydes, might be involved in the biochemical mechanism causing alcohol addiction (Fig. 1). If they are really formed endogenously in elevated concentrations, they might act centrally to promote a mechanism controlling alcohol drinking behaviour [1]. There are controversial reports on the detection of TIQs in mammalian tissues and fluids after ethanol intake [2–5]. Poor assay specificity and possible artifactual formation of these alkaloids during sample work-up and storage have been suggested to be responsible for these differences.

It was shown that simple TIQs, such as salsolinol (SAL), derived from the condensation of dopamine with acetaldehyde (a metabolite of ethanol), and salsoline (SALN), the 7-O-methylated product of SAL, are excreted not only by alcoholics but also by non-alcoholic (control) subjects, indicating the existence of normal endogenous TIQ sources [6]. Otherwise,

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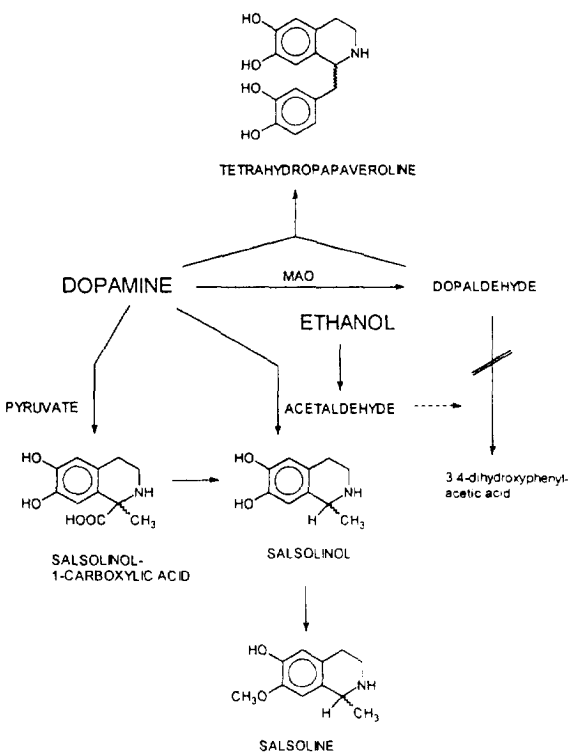


Fig. 1. Possible pathway for the endogenous formation of TIQ compounds.

TIQs are also present in some food and alcoholic beverages such as beer and wine [7].

TIQ compounds with a substituent in position 1 are chiral. Two stereoisomers with (*R*)-(+)- and (*S*)-(−)-configuration may be formed by the cyclization of dopamine with aldehydes. Different amounts of the enantiomers in biological samples would therefore indicate the participation of an enzymatic reaction in the biosynthesis of TIQs.

In all instances an appropriate analytical method is necessary to control the enantiomeric purity of the endogenously formed TIQs.

All known methods determining the proportion of the enantiomers of SAL in urine or plasma are based on high-performance liquid chromatographic (HPLC) separation with electrochemical detection. The *R*-enantiomer of SAL was found to be predominant in the urine [6] as well as in the plasma of healthy subjects [8], while in alcoholics mainly the *S*-enantiomer was found [9]. However, the large amounts of compounds similar to the analytes have not allowed, in our opinion, fair separation of salsolinol (especially of the *S*-enantiomer) from other compounds which complicate the quantification [10].

A sensitive and reliable gas chromatographic (GC) method would be more useful for the determination of enantiomers. Using gas chromatography–mass spectrometry (GC–MS), not only peak detection but also peak identification can be achieved. The lack of analytical techniques for the resolution of TIQ enantiomers has prompted us to seek a highly selective and sensitive method. We recently reported a new method for the quantitative analysis of racemic TIQs by GC–MS utilizing phase transfer-catalyzed extractive derivatization as a rapid sample clean-up procedure [11]. However, the application of different chiral columns did not yield a satisfactory separation of the TIQ enantiomers.

We now describe a new assay for the chromatographic separation of several TIQs. To achieve optimal resolution of the enantiomers, we decided to use GC–MS with a common non-chiral capillary column through chiral derivatizing agents. In addition, we applied this method to the quantification of the enantiomers of SAL in the urine and plasma of healthy subjects. Special dietary conditions were followed in this study to avoid interferences by SAL from foodstuffs.

2. Experimental

2.1. Chemicals and derivatization procedure

R-(+)-SAL and *S*-(−)-SAL were prepared as described previously [12]; the enantiomers of SALN and tetrahydropapveroline (THP) were synthesized according to Ref. [13]; (±)-norreticulin and (±)-SAL-1-carboxylic acid were gifts from Prof. Dr. M. Collins (Loyola University, Chicago, IL, USA). Racemic deuterated SAL (the internal standard) was prepared by treating 2,5,6-[$^2H_3$]dopamine (obtained from IC Chemikalien, Munich, Germany) with acetaldehyde as described recently [11]. (*R*)-(−)-2-

Phenylbutyryl chloride was prepared by treating butyryl chloride with thionyl chloride [14]. The product was distilled under vacuum and dissolved in chloroform. A 0.1 *M* solution was used for the derivatization of the TIQs. N-Trifluoracetylprolyl chloride was purchased as a 0.1 *M* solution in chloroform from Aldrich (Steinheim, Germany). N-methyl-N-trimethylsilyltrifluoracetamide (MSTFA) was purchased from Macherey–Nagel (Düren, Germany). Phenylboronic acid solid phases (PBA) were purchased from Varian (Harbor City, CA, USA). All other chemicals were of analytical grade from Baker (Groß Gerau, Germany).

The racemic TIQs were dissolved in methanol (100 ng/ml of each) and then evaporated to dryness under a stream of nitrogen. The residue was derivatized with 100 μl of MSTFA (30 min, 70°C). After this reaction time 100 μl of (*R*)-(−)-2-phenylbutyryl chloride or N-TFA-L-prolyl chloride were added. The mixture was shaken, and 1 μl was injected into the GC–MS system.

2.2. Sample collection and extraction

Plasma sample

Blood samples were centrifuged immediately at 1000 *g* for 10 min at 4°C; the plasma samples (5 ml) were acidified with 1 *M* perchloric acid (1:9, v/v), containing 0.01% ethylene glycol–bis(β-aminoethyl ether)–N,N,N′,N′-tetraacetic acid (EGTA), 0.01% semicarbazide hydrochloride and 0.02% sodium metabisulfite. Deuterated SAL (1 ng/ml in plasma) was added as internal standard. The samples were centrifuged at 1000 *g* for 10 min at 4°C, the protein was removed, and the supernatant was heated for 30 min at 80°C to hydrolyze any conjugated SAL. The supernatant was used for the extraction procedure.

Urine sample

Aliquots containing 5 ml of urine sample were used for the analyses. Deuterated SAL (5 ng) was added as internal standard, and the urine was worked up in the same manner as described above.

2.3. Extraction of SAL from biological samples

PBA cartridges were used for the solid-phase extraction of SAL. The cartridges were conditioned by washing twice with 1 ml of methanol, 1 ml of water and 1 ml of saturated $NaHCO_3$ buffer solution (pH 9).

The sample was brought to pH 6.5–7 by adding KOH buffer, and the insoluble perchlorate was filtered off. The sample buffered at pH 9 with $NaHCO_3$ was loaded on to the PBA cartridge and passed through by gentle low-pressure aspiration. After washing twice with 1 ml of water and 2 ml of methanol, the adsorbed SAL was eluted from the cartridge with 1 ml of 5 *M* formic acid–methanol (1:5). The sample was evaporated to dryness and derivatized as described above with 100 μl of MSTFA and 100 μl of (*R*)-(−)-2-phenylbutyryl chloride. Immediately prior to injection into the GC–MS system, excess reagents were removed under dry nitrogen and the residue was redissolved in 20 μl of dry chloroform.

2.4. Calibration curves

Standard curves were prepared for each SAL enantiomer by adding varying known amounts (250–5000 pg) of *R*- and *S*-SAL and fixing 5 ng of the deuterated SAL to 5 ml of water and carrying out the solid-phase extraction described above in triplicate. The derivatized samples were evaporated to dryness under a stream of dry nitrogen. The residue was dissolved in 20 μl of dry chloroform, and a 2-μl aliquot was immediately subjected to GC–MS analysis [in the selected-ion monitoring (SIM) mode]. Standard curves were generated by least-squares linear regression analysis.

2.5. Studies of artifactual condensation, racemization and hydrogen–deuterium (H–D) exchange

A 5-ng amount of deuterated dopamine was added to 5 ml of urine and 5 ml of plasma without deuterated standard, and the mixture

was worked up as described above and analyzed by GC–MS for the presence of deuterated SAL.

R-SAL (10 ng) was added to 5 ml of water, and the mixture was taken through the whole procedure and analyzed by GC–MS for the presence of *S*-SAL. This experiment was repeated with 100 ng of *R*-SAL using plasma and urine samples (1 ml of each).

To ascertain that the deuterated standard was stable in an aqueous medium and that there was no H–D exchange during the sample preparation, deuterated SAL (1 ng/ml) was added to 5 ml water, and the sample was worked up as described and analyzed for the presence of non-deuterated salsolinol.

2.6. Precision and recovery of the method

To study the reproducibility of the method, 5 ml of a urine sample (500 ml) of a single subject were repeatedly worked up ($n = 5$) on the same day as well as on different days. The samples were analyzed and the coefficients of variation (C.V.) determined for *R*- and *S*-SAL.

For the determination of the extraction efficiency, deuterated SAL (1 and 2 ng) was added to 5 ml of urine and 5 ml of plasma. The samples ($n = 6$) were worked up as usual, and the recovery of deuterated SAL was calculated by comparing the mean deuterated peak areas in all extracted samples with the mean deuterated peak areas in a series of non-extracted derivatized deuterated SAL.

The limit of quantification of *R*- and *S*-SAL was determined by adding 200 pg of deuterated SAL and different amounts (70, 100 and 200 pg) of *R*-SAL and *S*-SAL to urine and plasma samples (1 ml of each) after the samples were allowed to stand at pH 12 for 24 h to remove any original SAL. The samples ($n = 5$) were worked up as usual and analyzed by GC–MS.

2.7. Instrumentation and chromatographic conditions

GC–MS resolution of the TIQ enantiomers was carried out with a Fisons Trio 1000 GC–MS data system. Derivatized samples were analyzed using a 30-m BGB-silaren capillary column (0.32 mm I.D. and 0.12 μm film thickness) from Chromtech (Hofheim am Taunus, Germany). Ultrapure helium was used as the carrier gas with a head pressure setting of 1 bar. The injector temperature was 300°C; interface and ion source temperatures were maintained at 300 and 250°C, respectively. The splitless injection mode was used; the purge valve was turned on 1.5 min after injection, with a split flow-rate of 25 ml/min during the GC run. The GC oven temperature was held at 200°C for 1 min and then programmed at 10°C/min to 330°C for 15 min. Electron-impact ionisation mass spectra were recorded in the full-scan mode for all TIQs. For the quantitative determination of the enantiomers of SAL from plasma and urine samples, the SIM mode was used. The ions monitored were m/z 469.22 and 454.41 for SAL and m/z 471.22 and 456.41 for the standard.

3. Results and discussion

Our approach consists in the use of (*R*)-(−)-2-phenylbutyryl chloride as a chiral derivatization agent, which produces diastereomers of the TIQs which can be baseline-resolved on a conventional GC column.

TIQs bearing a secondary amino group and hydroxy substituents form volatile compounds after the two-step derivatization with MSTFA and (*R*)-(−)-2-phenylbutyryl chloride.

The separation of several TIQs and the resolution of the enantiomers are shown in Fig. 2 and Table 1. The analysis of each single derivatized enantiomer of SAL, SALN and THP revealed that the *S*-enantiomer of the TIQs was eluted before the *R*-enantiomer. Fig. 3 shows the electron-impact mass spectra of the derivatized TIQs.

A solid-phase extraction on PBA columns was used as the clean-up procedure for the quantification of the enantiomers of SAL from biological samples. Fig. 4 shows a chromatogram obtained from the plasma of a healthy subject under special dietary conditions to avoid interferences by SAL in foodstuffs. Utilizing the SIM mode,

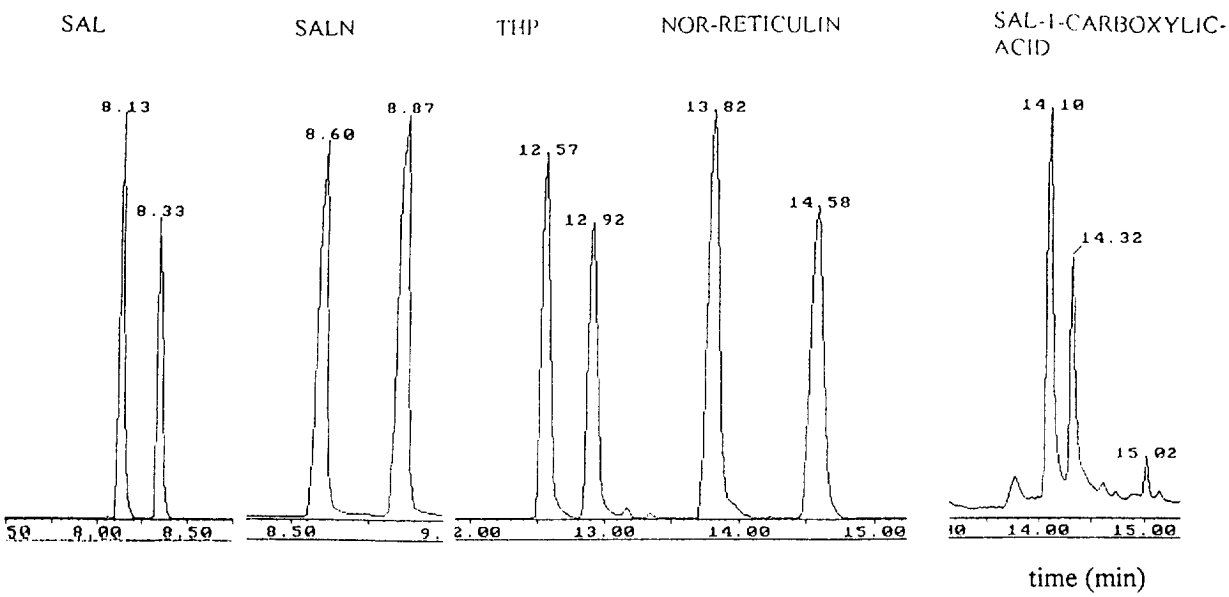


Fig. 2. GC–MS profiles of TIQ enantiomers which were transformed to diastereomers by derivatization with MSTFA and *R*-(−)-2-phenylbutyryl chloride.

SAL is the main peak observed in the chromatograms obtained from plasma or urine samples. Peak detection was also obtained by measuring in the full-scan mode when 30 ml of urine were used. In this way the recording of the complete mass spectra confirming the chemical structure of SAL was possible.

Table 2 shows a summary of the statistical data on the analyses of the SAL enantiomers.

The correlation coefficients obtained for the calibration curves (50–1000 pg/ml) were consistently higher than 0.99. The C.V.s obtained from the repeated urine analysis of one subject were always less than 6%, both for the intra-day ($n = 5$) and the inter-day ($n = 5$) experiments. The limit of quantification, defined as the concentration for which the standard deviation of five determinations is within ± 10% of the mean value and the C.V. less than 10%, was found to be 100 pg/ml for each SAL enantiomer. The limit of detection was found to be at least 50 pg/ml.

Because the pure derivatized deuterated SAL was not available, recovery was calculated at two

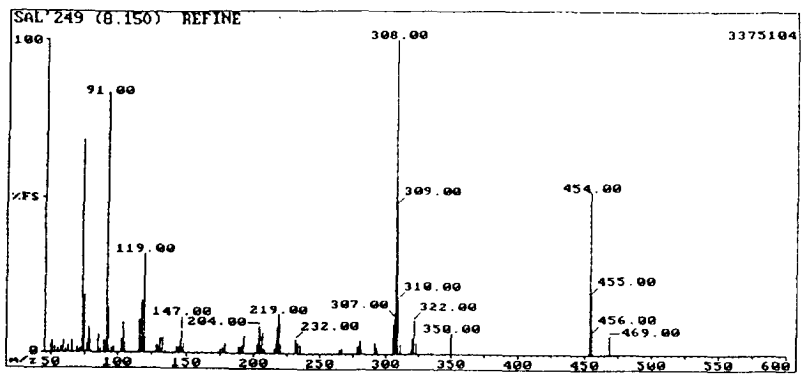
Table 1
Retention times (t_R), resolutions (R_s), GC programme (starting temperature always 200°C, final temperature always 330°C) and *S*/*R* ratios of racemic TIQs after derivatization and analysis by GC–MS

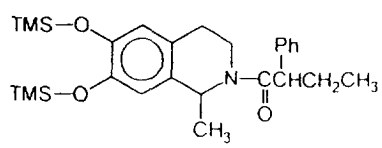
TIQ	t_R (*S*; *R*)	R_s^a	GC programme	*S*/*R* ratio
Salsolinol (SAL)	8.23; 8.45	3.24	10°C/min	1.14
Salsoline (SALN)	8.60; 8.87	5.60	10°C/min	0.90
Salsolinol-1-carboxylic acid	14.10; 14.32	3.23	7°C/min	1.35
Tetrahydropapaveroline (THP)	12.60; 12.95	2.57	10°C/min	1.09
Norreticuline	13.82; 14.58	4.85	10°C/min	1.19

[a] $R_s = 1.177(t_{RR} - t_{RS})/(w/2_R + w/2_S)$.

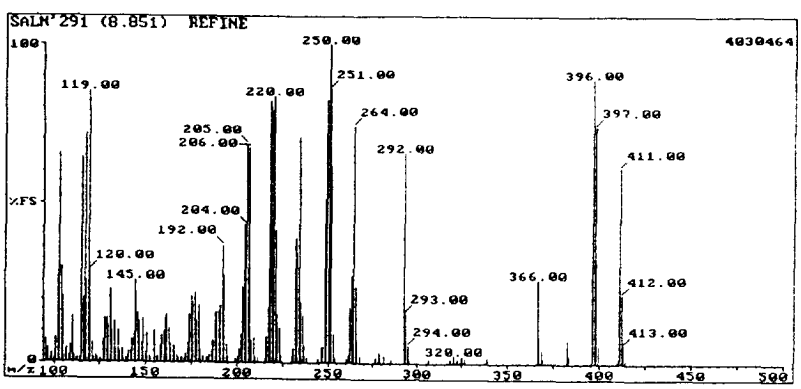
concentrations by dividing mean areas ($n = 5$) obtained after complete extraction and derivatization of plasma and urine samples containing 0.4 and 0.2 ng/ml deuterated SAL by mean areas obtained after direct derivatization of the same quantities of the pure deuterated standard. The recovery (at least 65%) of the method is low, mainly due to TIQ oxidation during the solid-phase extraction, but was found to be satisfactory for the quantitative determination of SAL enantiomers from plasma and urine samples.

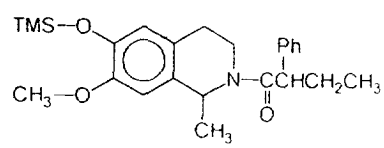
Deuterated SAL (98% *d*-SAL) was used as internal standard for the quantification of SAL. There was no change of the 2% unlabelled amount of deuterated SAL even after a one-month storage of an aqueous deuterated SAL



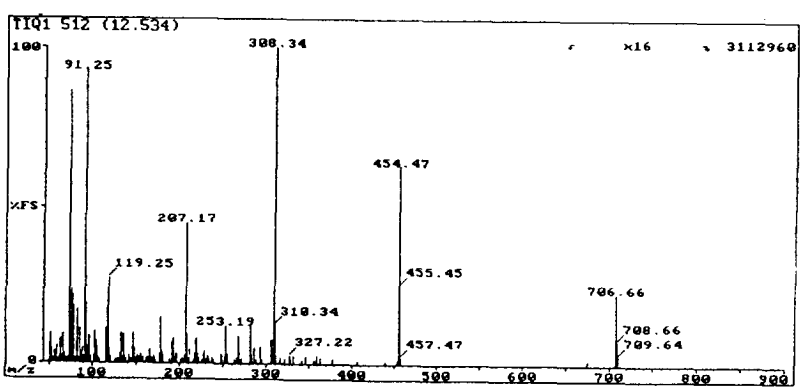


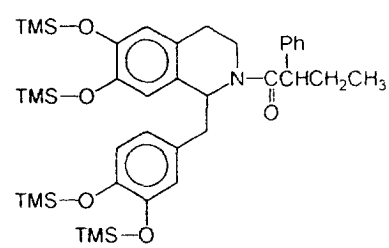
Salsolinol





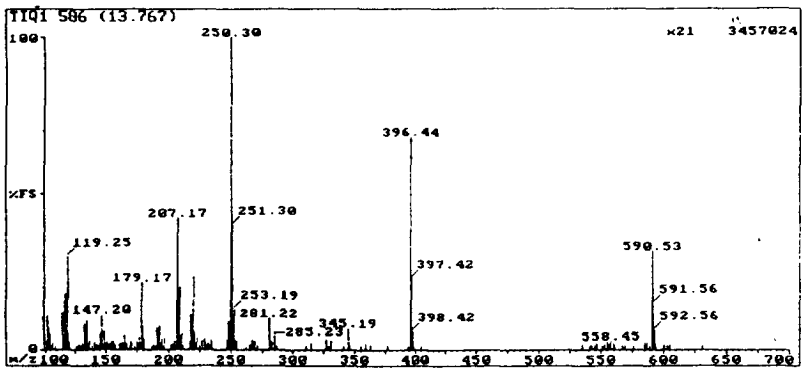
Salsoline

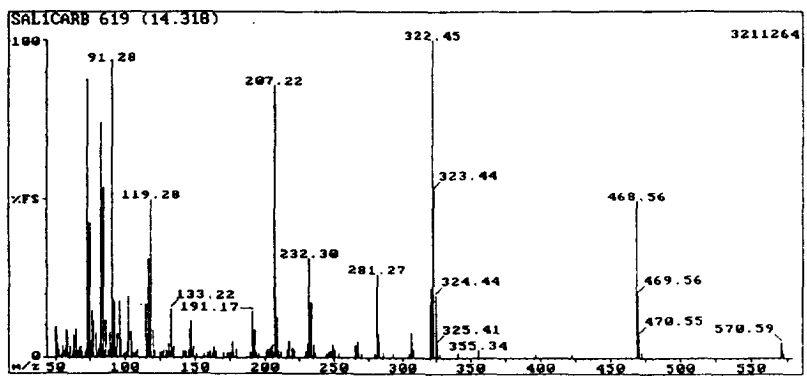


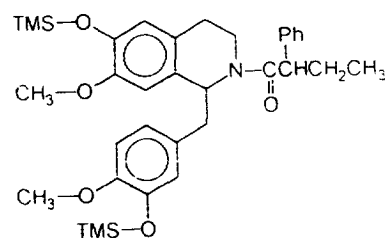


Tetrahydropapaveroline

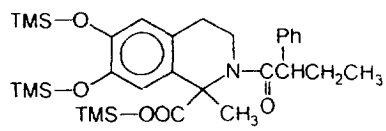
Fig. 3.







Nor-Reticuline



Salsolinol-1-carboxylic acid

Fig. 3. Electron-impact mass spectra of the derivatized TIQs corresponding to those of Fig. 2.

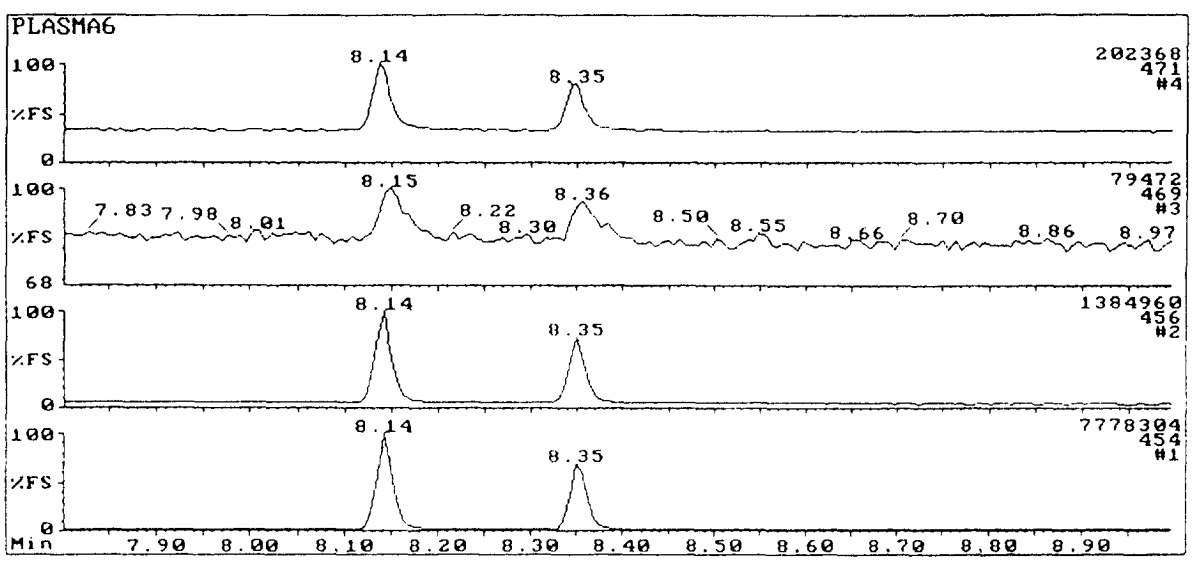


Fig. 4. SIM chromatogram of SAL enantiomers after extraction from a plasma sample of a healthy subject and derivatization with MSTFA and *R*-(−)-2-phenylbutyryl chloride.

Table 2
Statistical data concerning the analysis of SAL enantiomers from plasma and urine

	S-SAL	*R*-SAL
Calibration		
Range (pg/ml)	50–1000	50–1000
Correlation coefficient	0.998	0.999
Recovery (n = 5)		
Theoretical values (pg/ml)	400	400
Measured values (mean ± S.D. in pg/ml) (C.V. in %)	258 ± 23 (8.9)	254 ± 19 (7.5)
Theoretical values (pg/ml)	200	200
Measured values (mean ± S.D. in pg/ml) (C.V. in %)	137 ± 16 (11.7)	136 ± 21 (15.4)
Within-day variation (n = 5) (single urine sample)		
Measured values (mean ± S.D. in pg/ml) (C.V. in %)	753 ± 39 (5.2)	842 ± 35.4 (4.2)
Day-to-day variation (n = 5) (single urine sample)		
Measured values (mean ± S.D. in pg/ml) (C.V. in %)	784 ± 37.6 (4.8)	811 ± 47.1 (5.8)
Limit of quantification (n = 5)		
Theoretical values (pg/ml)	100	100
Measured values (mean ± S.D. in pg/ml) (C.V. in %)	102 ± 8.2 (8.0)	97 ± 7.3 (7.5)

solution as measured by GC–MS, indicating that the deuterated standard was stable. At the level of deuterated standard added to each sample, there is no quantification problem associated with the unlabelled part of the standard. The described sample work-up procedure does not lead to any H–D exchange. We have also established that the extraction and derivatization procedure does not lead to artifactual SAL formation.

Surprisingly, the results obtained by others who detected only the *R*-SAL in healthy subjects by using HPLC with electrochemical detection [6,8] were not confirmed by our GC–MS method. SAL was found racemic in all plasma and urine samples.

In order to prove our results, we used another chiral reagent (N-TFA-L-prolyl chloride) for the separation of the enantiomers of SAL from biological samples. The derivatization with this reagent yields diastereomers showing a different retention time compared with that of the 2-phenylbutyryl chloride derivatives as confirmed by the analyses. However, the separation of SAL was not baseline-resolved; the SAL found in plasma or urine is racemic in healthy subjects.

Obtaining high specificity was the major consideration during assay development. HPLC methods, even in conjunction with electrochemical detection, are not sufficiently specific to allow selective detection and quantification of SAL enantiomers. In biological matrices, co-extraction of unknown compounds can lead to a complex chromatogram and often to overlap between the analyte and interfering peaks. Accurate quantification of the enantiomers of SAL necessitated the incorporation of a deuterated internal standard. GC–MS with deuterated SAL as a standard and occurrence of peaks at m/z 469 and 455 with the appropriate ion abundance ratio (m/z 454/469) were all necessary criteria for the identification of SAL.

This method allows the enantiomeric resolution of different TIQs and offers a good possibility for the analysis of TIQ enantiomers from biological samples.

A nearly racemic mixture of SAL in urine or plasma samples of healthy subjects does not exclude an enzymatic pathway for the endogenous SAL formation. Further analytical investigations utilizing the method described here in biological samples containing high amounts of

free dopamine, the precursor of SAL (e.g. brain samples), should be carried out to clarify the endogenous formation of SAL and other TIQs.

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