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Gas chromatographic–mass spectrometric procedures for determination of the catechol-*O*-methyltransferase (COMT) activity and for detection of unstable catecholic metabolites in human and rat liver preparations after COMT catalyzed *in statu nascendi* derivatization using *S*-adenosylmethionine

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Abstract

A procedure is presented for determination of the catechol-*O*-methyltransferase (COMT) activity in liver cytosolic preparations using 3,4-dihydroxyphenethylamine as substrate and by quantifying the product 3-methoxy-4-hydroxyphenethylamine (3-MHP). For quantification of 3-MHP in liver cytosolic preparations a gas chromatographic–mass spectrometric procedure after liquid–liquid extraction and acetylation was established and validated. The intra- and inter-day accuracy and precision were better than 15% and 20%, respectively. Extraction efficiency and selectivity were also sufficient. For *in statu nascendi* derivatization of unstable catecholic metabolites in liver microsome preparations, cytosolic preparations with COMT activities of at least 1 nmol product/min/mg protein were used after addition of *S*-adenosylmethionine. Such catecholic metabolites, which are claimed to be responsible for toxic effects in vivo, e.g., neurotoxicity or carcinogenesis, must not be overlooked in in vitro metabolism studies. Using this trick, gas chromatography–mass spectrometry (GC–MS) was suitable for the determination of catecholic metabolites in human and rat liver preparations after the same sample preparation as for 3-MHP quantification. The applicability was exemplified for the antidepressant paroxetine.

Keywords: Enzymes; Catechol-*O*-methyltransferase; 3,4-Dihydroxyphenethylamine

1. Introduction

Studies on the metabolism of xenobiotics are often performed using subcellular fractions of animal or human hepatocytes (e.g., microsomal and/or cytosolic preparations). Identification of the formed metabolites is usually performed by gas chromatography–mass spectrometry (GC–MS) or liquid chromatography (LC)–MS. Detection of catechols (*ortho*-diphenols) formed in microsome preparations is difficult. In such in vitro preparations, catechols are not conjugated as under in vivo conditions, unless phase II cosubstrates were added. Free catechols are rather unstable due to oxidation [1], so that they must be stabilized during incubation. From the

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toxicological point of view, such catechols must not be overlooked, since they are claimed to be responsible for toxic effects, e.g., neurotoxicity [1] or carcinogenesis [2]. Therefore, we tried to derivatize the catechols *in statu nascendi* to stable hydroxy methoxy analogues by addition of *S*-adenosylmethionine (SAM) and cytosol containing catechol-*O*-methyltransferase (COMT; EC 2.1.1.6). This trick has been suitable for identification of unknown catecholic metabolites in liver microsome preparations [3,4]. However, the COMT activity in the cytosol must first be determined to ensure efficient and reproducible derivatization. High-performance liquid chromatography (HPLC) procedures like that of De-Santi et al. [5] have not been used, since we tried to use the same GC–MS procedure for determination of the COMT activity and for the identification of the metabolites.

Therefore, in the following, a validated GC–MS procedure will be presented for quantification of 3-methoxy-4-hydroxyphenethylamine (3-MHP) in liver cytosolic preparations after liquid–liquid extraction and acetylation. The application of this procedure will be shown for the determination of the COMT activity in liver cytosolic preparations. The use of such cytosolic preparations with sufficient COMT activity will also be presented for *in statu nascendi* derivatization of unstable catecholic metabolites in liver microsome preparations after addition of SAM. This procedure will be exemplified for GC–MS detection of metabolites of the antidepressant paroxetine.

2. Experimental

2.1. Chemicals and reagents

3,4-Dihydroxyphenylethylamine (3,4-DHP, dopamine, 3-hydroxytyramine), 3-methoxy-4-hydroxyphenylethylamine (3-MHP, 3-methoxytyramine), methaqualone and *S*-adenosylmethionine (SAM) were obtained from Sigma–Aldrich (Deisenhofen, Germany). All other chemicals were of analytical or biochemical grade and were obtained from Merck (Darmstadt, Germany).

2.2. Preparation of microsomal and cytosolic fractions

Adult male Wistar rats were obtained from Charles River (Sulzfeld, Germany). Human liver samples were obtained from liver carcinoma resections after obtained written consent. Liver samples were homogenized in two volumes of 1.15% KCl solution. Microsomes and cytosol were isolated after 10 000 *g* and 100 000 *g* centrifugation. The microsomes were resuspended in 1.15% KCl solution for washing and again centrifuged at 100 000 *g*. The microsome pellets and the cytosol samples were stored at −80°C before incubation. Microsomal and cytosolic protein concentrations were determined by the Bio-Rad protein assay kit (Bio-Rad, Munich, Germany) [6] using a bovine serum albumin standard solution. Total cytochrome P-450 levels were determined according to Omura and Sato [7].

2.3. Assay for the quantification of 3-MHP in cytosol

3-MHP was added to aliquots of the cytosolic fraction (1–1.5 mg of protein) to reach final concentrations of 0.25, 4.9, 9.8, 19.6 or 39.2 μ*M* for the calibrators or of 0.25, 9.8 or 39.2 μ*M* for the quality control (QC) samples. SAM (0.58 m*M*) and $MgCl_2$ (5 m*M*) solved in 0.1 *M* phosphate buffer (pH 7.4) were added to reach a final volume of 1 ml.

2.4. Assay for the determination of the COMT activity in cytosol

3,4-DHP (264 μ*M*) was incubated with aliquots of the cytosolic fraction (1–1.5 mg protein), SAM (0.58 m*M*) and $MgCl_2$ (5 m*M*) in 0.1 *M* phosphate buffer (pH 7.4, final volume 1 ml) for 2, 4, 6 or 8 min at 37°C. The reaction was stopped by adding the organic extraction mixture (cf. Section 2.6).

2.5. Assay for the determination of catecholic metabolites in microsome preparations

Aliquots of the microsome fraction (1.5 mg protein/ml) were incubated with substrate (3 μl methanolic or aqueous stock solution, 10 mg/ml), 1.2 m*M*

NADP, 2 U isocitrate dehydrogenase, 5 m*M* isocitrate, 5 m*M* $MgCl_2$, cytosolic preparation (1.5 mg protein/ml) and 0.58 m*M* SAM in 0.1 *M* phosphate buffer (pH 7.4, final volume 1 ml) for 90 min at 37°C. Again, the reaction was stopped by adding the organic extraction mixture.

2.6. Sample preparation

The whole incubation mixture (cf. Sections 2.3, 2.4 or 2.5) was mixed with 10 ml of a dichloromethane–isopropanol–ethyl acetate (1:1:3, v/v/v) mixture and with 100 μl of methanolic methaqualone solution (0.01 mg/ml) as internal standard (I.S.). A mixture of 1.5 ml of 37% hydrochloric acid, 2.5 ml of 2.3 *M* aqueous ammonium sulphate and 2 ml of a 10 *M* aqueous sodium hydroxide solution was added to obtain a pH between 8 and 9 according to Ref. [8]. After cooling on ice, the sample was shaken. After phase separation by centrifugation, the organic layer was transfered and evaporated to dryness, and the residue was acetylated with 100 μl of an acetic anhydride–pyridine (3:2, v/v) mixture for 2 min under microwave irradiation [9]. After evaporation, the residue was dissolved in 100 μl of methanol and 2 μl of this solution was injected into the gas chromatograph.

2.7. Apparatus

The extracts were analyzed using a Hewlett-Packard (HP, Waldbronn, Germany) 6890A gas chromatograph combined with an HP 5972 MSD mass spectrometer and an HP Chemstation Series 1701AA. The GC conditions were as follows: splitless injection mode; column, HP-1 capillary (12 m×0.2 mm I.D.), cross linked methylsilicone, 330 nm film thickness; injection port temperature, 280°C, carrier gas, helium; flow-rate 1 ml/min; column temperature, programmed from 100 to 310°C at 30°C/min, initial time 3 min, final time 8 min. The MS conditions were as follows: full scan mode or single ion monitoring (SIM) mode; electron impact (EI) ionization mode: ionization energy, 70 eV; ion source temperature, 220°C; capillary direct interface heated at 260°C.

2.8. GC–MS procedure

For quantification of 3-MHP, the SIM mode was used with the following selected ions: m/z 137, 150 and 209 for 3-MHP (time window 8.0–8.3 min) and m/z 233, 235 and 250 for the I.S. methaqualone (time window 8.3–8.8 min).

For detection and identification of unknown catecholic metabolites in the microsome incubates, the full scan mode was used. The probable presence of the *in statu nascendi* methylated catechol metabolites could be detected using mass chromatography with ions selected from the mass spectrum of the (acetylated) parent drug and with ions calculated from these ions taking into consideration the metabolic modification of the molecule. The metabolites indicated in the reconstructed mass chromatogram could be identified by interpretation of the underlying EI mass spectra in correlation to that of the known parent compound according to the rules described by McLafferty and Turecek [10].

2.9. Calibration, precision, accuracy, extraction efficiency and selectivity of the 3-MHP quantification

A calibration curve was constructed for 3-MHP at five concentrations ranging from 0.25 μ*M* to 39.2 μ*M*. QC samples of 3-MHP were independently prepared. The final concentrations of low, medium and high QC samples were 0.25, 9.8 and 39.2 μ*M*. The robustness of the method was assessed by analysis of 15 QC samples (five of each concentration) on a single assay day to determine the intra-day accuracy and precision. Inter-day accuracy and precision were determined by analysis of six QC samples (two each of low, medium and high concentration) on four different assay days. Extraction efficiency of 3-MHP was assessed at 4.9 and 39.2 μ*M*. The peak area ratio of extracted 3-MHP and methaqualone (added after extraction) was compared to that of 3-MHP spiked in extracted cytosolic matrix and methaqualone. Linear regression analysis of the peak area ratios of 3-MHP to internal standard methaqualone against nominal concentrations was performed to determine the slope, intercept and coefficient of determination (r^2). The selectivity of

the method was checked by analysis of extracts of cytosolic incubates from five different liver samples without substrate 3,4-DHP and product 3-MHP (blank).

3. Results and discussion

The first step of our studies was the development of a validated GC–MS procedure for quantification of 3-MHP in liver cytosolic preparations. This procedure was necessary for the determination of the cytosolic COMT activity. 3,4-DHP was used as substrate and the product 3-MHP was quantified. 3,4-DHP was selected for COMT activity determination, since it was commercially available as well as 3-MHP. In Fig. 1, the corresponding COMT catalyzed reactions are shown. The 4-methylated product (4-MHP) was formed only in minor amounts and did not disturb the quantification of 3-MHP. Fig. 2 shows the EI mass spectra and the structures of the two acetylated products and of our routinely used I.S. methaqualone. From these mass spectra the ions were selected for SIM quantification.

Cytosolic preparations with sufficient COMT activity were used for *in statu nascendi* derivatization of unstable catecholic metabolites in liver microsome preparations after addition of SAM. Using this trick, GC–MS was suitable for the determination of catecholic metabolites formed by microsomal enzymes after COMT catalyzed methylation, extraction and acetylation. Acetylation was necessary to improve the GC properties of the analytes. The extraction pH and solvent used have proved to be very efficient in extracting compounds with very different chemical properties from biomatrices [8,11–15].

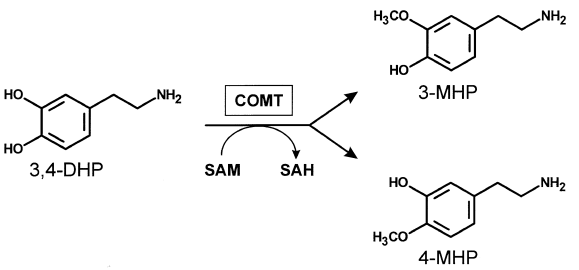


Fig. 1. Methylation of 3,4-dihydroxyphenethylamine (3,4-DHP) to 3-methoxy-4-hydroxyphenethylamine (3-MHP) or to 4-methoxy-3-hydroxyphenethylamine (4-MHP) by *S*-adenosylmethionine (SAM) catalyzed by COMT.

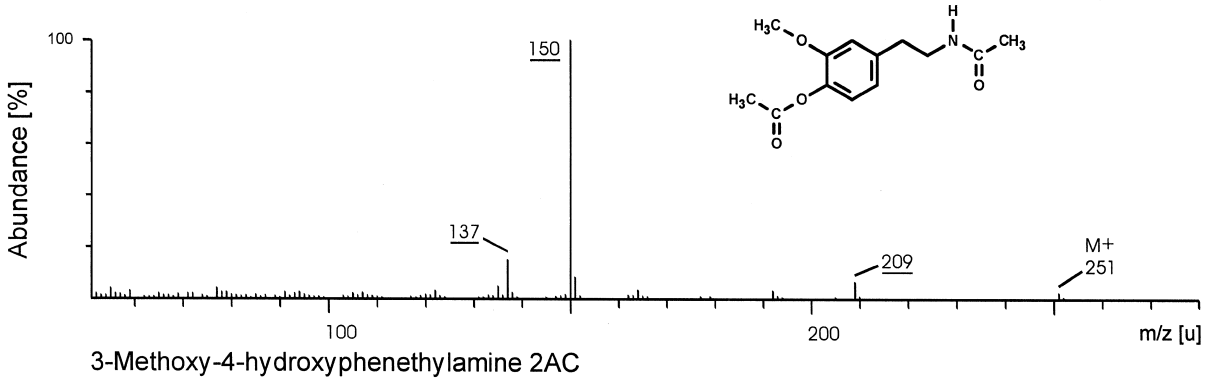
3.1. Quantification of 3-MHP in liver cytosol

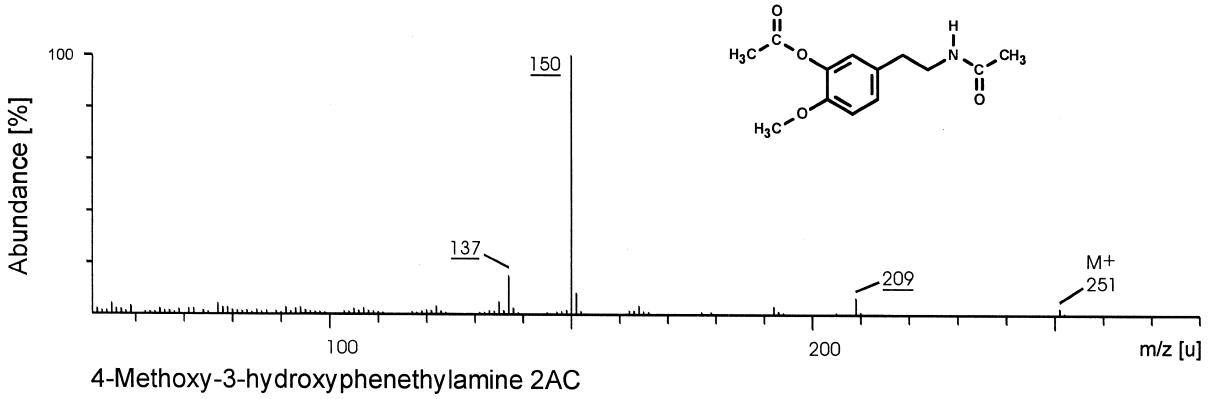
The aim of these studies was to use standard GC–MS and sample preparation procedures for quantification of 3-MHP, which were approved for metabolism studies [8,13,14]. Fig. 3 shows mass fragmentograms of the ions m/z 137, 150 and 209 (time window 8.0–8.3 min) and of the ions m/z 233, 235 and 250 (time window 8.3–8.8 min) for indication of the presence of acetylated 3-MHP and the I.S. methaqualone in an acetylated extract of a blank cytosolic preparation (upper part) and of a cytosolic preparation spiked with 9.8 μ*M* 3-MHP (QC,

Table 1
Intra- and inter-day accuracy and precision of the determination of 3-methoxy-4-hydroxyphenethylamine in cytosol

	Actual concentration (μ*M*)	Mean calculated concentration (μ*M*)	Precision (%)[a]	Accuracy (%)[b]
Intra-day (n=5)				
Low QC	0.25	0.29	11.9	14.7
Medium QC	9.8	9.71	4.6	−0.9
High QC	39.2	40.51	6.5	3.1
Inter-day (n=8)				
Low QC	0.25	0.2	10.7	−19.6
Medium QC	9.8	9.4	10.3	−4.1
High QC	39.2	45.4	13.8	15.9

[a] Precision=(SD/mean)×100.
[b] Accuracy=[(mean calculated concentration−actual concentration)/actual concentration]×100.





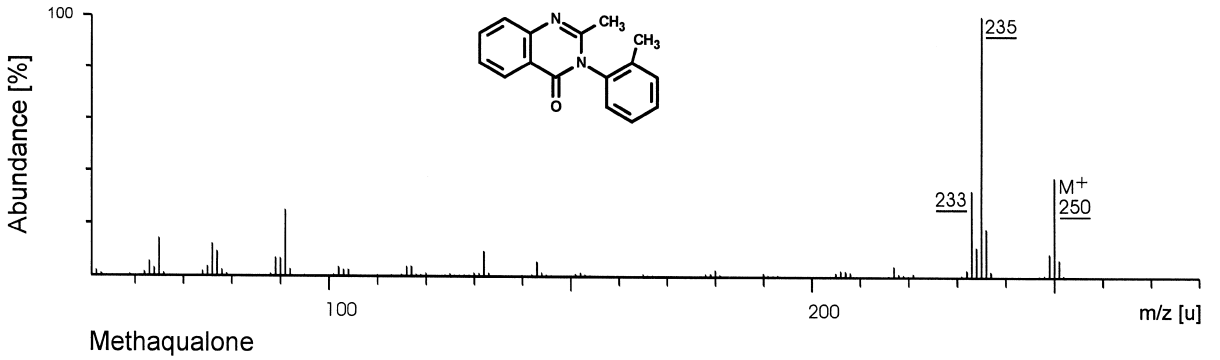


Fig. 2. Mass spectra and structures of acetylated 3-MHP, 4-MHP and methaqualone. The ions used for SIM quantification are underlined.

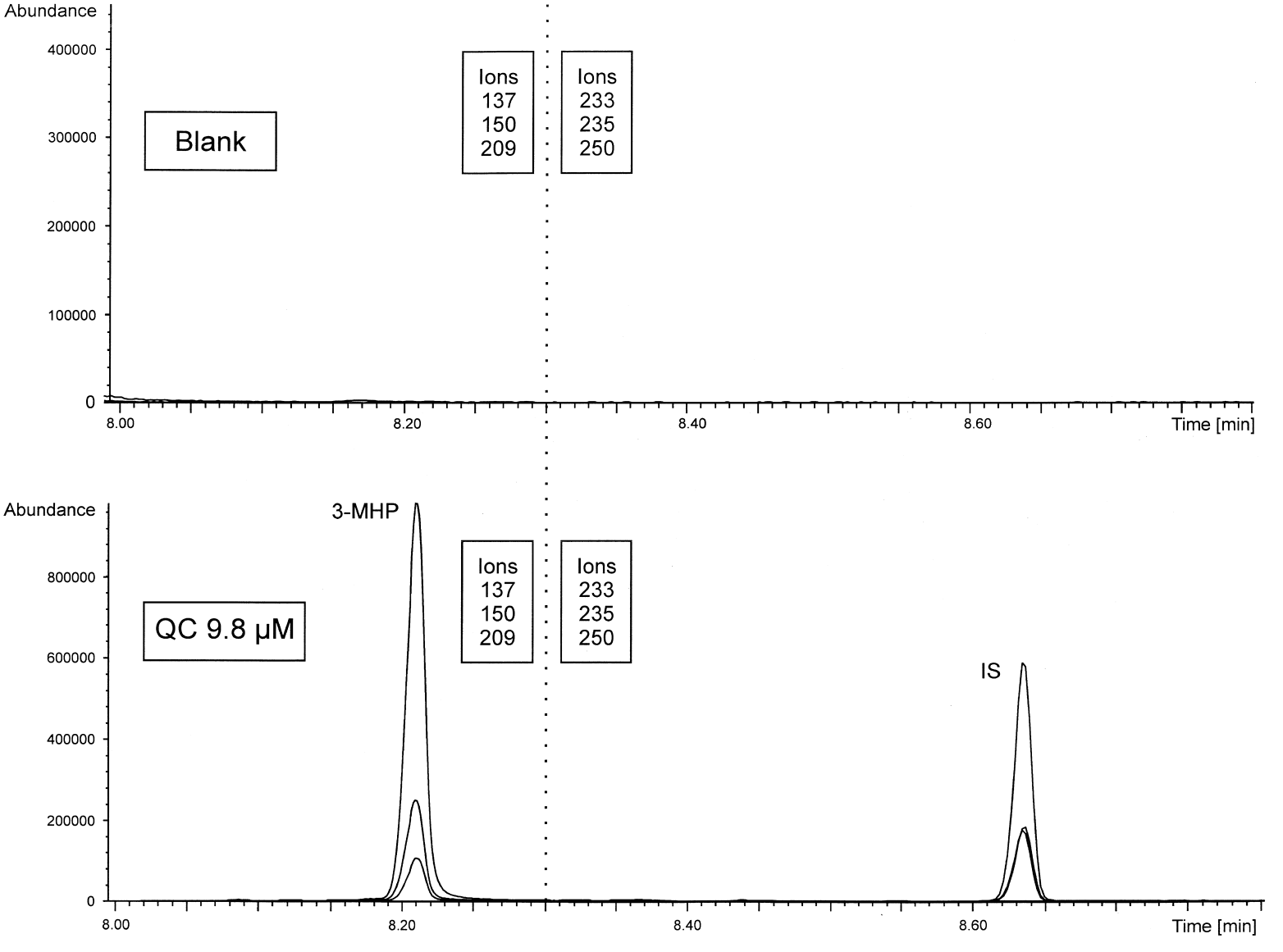


Fig. 3. Mass fragmentograms of the ions m/z 137, 150 and 209 (time window 8.0–8.3 min) and of the ions m/z 233, 235 and 250 (time window 8.3–8.8 min) for indication of the presence of acetylated 3-MHP and the I.S. methaqualone in an acetylated extract of a blank cytosolic preparation (upper part) and of a cytosolic preparation spiked with 9.8 μM 3-MHP (QC, medium) and the I.S.

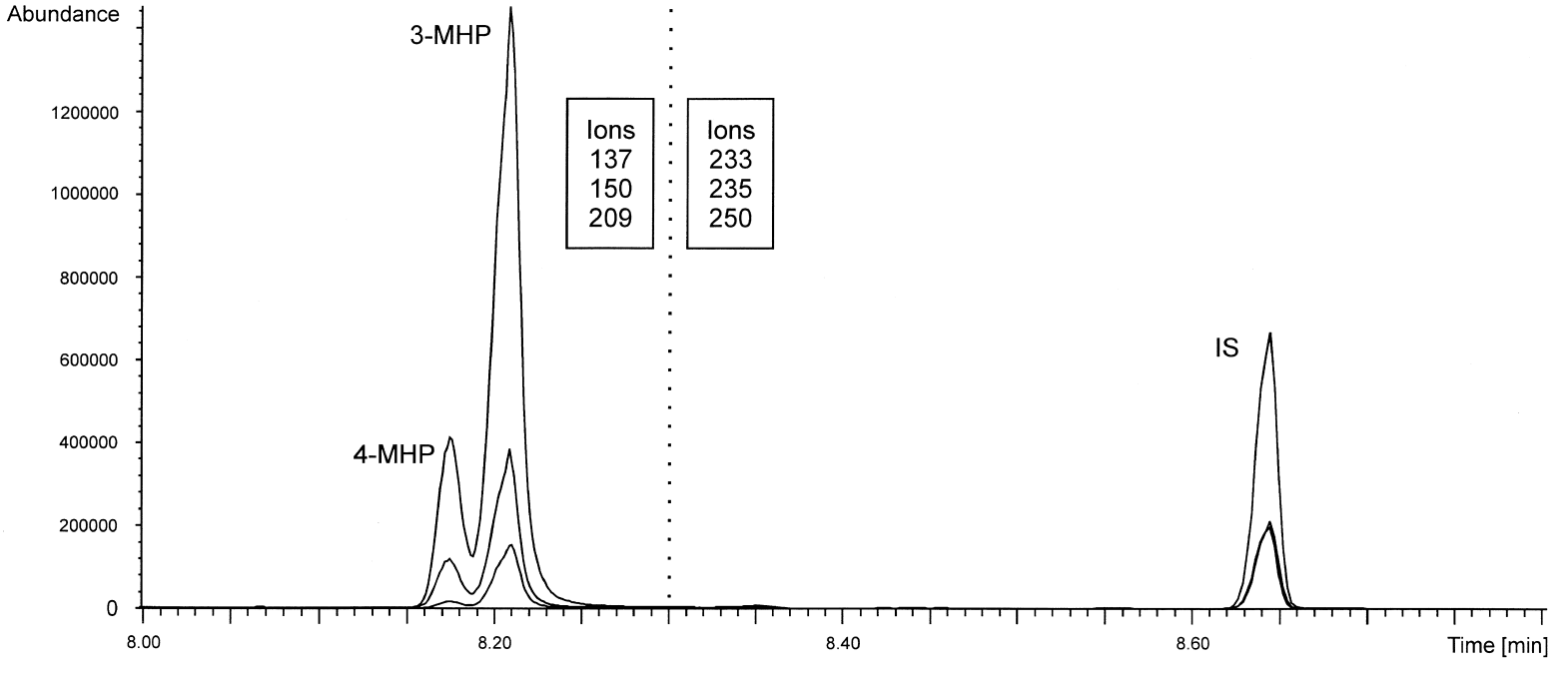


Fig. 4. Mass fragmentograms of the ions m/z 137, 150 and 209 (time window 8.0–8.3 min) and of the ions m/z 233, 235 and 250 (time window 8.3–8.8 min) for indication of the presence of acetylated 3-MHP, 4-MHP and the I.S. methaqualone in an acetylated extract of a cytosolic preparation spiked with 3,4-DHP and incubated with SAM for 8.0 min.

medium) and the I.S. As shown, no interfering peak appeared in the blank sample and the peaks of 3-MHP and of the I.S. are sharp. The results of the validation of this method were as follows. The calibration curve was linear from 0.25 to 39.2 μ*M* with mean r^2 values of 0.990 (n=5). The lower to middle and the middle to upper point of slope deviated less than 5% from the overall slope as claimed by [16]. The accuracy and precision data for the quantification of the QC samples are shown in Table 1. The extraction efficiency was determined at concentrations of 4.9 and 39.2 μ*M* (n=5). The overall mean (±SD) percentage extraction efficiencies were 40.6±17.3 and 34.5±9.2, respectively. The rather low extraction efficiencies were acceptable, taking into consideration, that a universal procedure was used, which was suitable for a wide range of (catecholic) metabolites [8,11–15].

3.2. Determination of the COMT activity in liver cytosol

The determination of the COMT activity in rat and human liver cytosolic preparations was necessary for the selection of such preparations for catechol derivatization as described in Section 3.3. As we have seen, COMT activity in human cytosol was less than that in rat cytosol probably due the sampling in the clinics. Therefore, we recommend to use rat cytosol for both, the rat and human liver microsome preparations. In our experience, the COMT activity determined using our assay should be at least 1 nmol product/min/mg protein to ensure sufficient derivatization rates [3,4,13,14]. In any case, the COMT activity should be determined prior to application. As already mentioned, the COMT catalyzed product 3-MHP was quantified after incubation of 3,4-DHP. Fig. 4a shows mass fragmentograms (same SIM method as described in Section 3.1) indicating the presence of 3-MHP, 4-MHP and the I.S. methaqualone in an acetylated extract of a cytosolic preparation spiked with 3,4-DHP and incubated with SAM for 8.0 min. The 4-methylated isomer was formed only in minor amounts, which was in accordance with the literature [17–19]. It did not disturb the determination of 3-MHP. Fig. 5 shows a representative time course of the formation of 3-MHP in rat liver cytosol incubations for the calculation of the

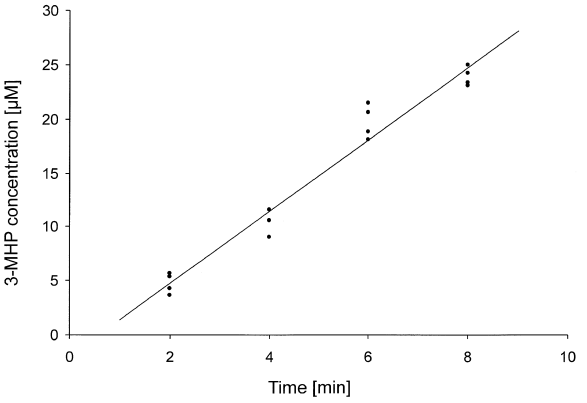


Fig. 5. Representative time course of the formation of 3-MHP in rat liver cytosol incubations for the calculation of the COMT activity (n=4). In this sample, the specific COMT activity was 2.2±0.11 nmol/min/mg protein.

COMT activity (n=4). The activity was calculated from the slope of the regression line. In this sample, the specific COMT activity was 2.2±0.11 nmol/min/mg protein. Typical activities for rat liver cytosol samples were about 1–3 nmol/min/mg protein, whereas in our human liver cytosol samples typical activities were about 0.3 nmol/min/mg protein. The coefficients of variation were less than 20%, showing that our procedure is suitable for determination of COMT activity. The determined activities are in accordance with the literature [5,17].

3.3. Detection of catecholic metabolites in liver microsome preparations

Rat liver cytosol preparations with COMT activities of at least 1 nmol product/min/mg protein were used for *in statu nascendi* derivatization of unstable catecholic metabolites in liver microsome preparations after addition of SAM. Metabolism studies showed that catechols formed by microsomal enzymes were indeed detected only after the addition of SAM and cytosol. Using this trick, GC–MS was suitable for the determination of catecholic metabolites in human and rat liver preparations after the same sample preparation as used for 3-MHP quantification. In the following, the applicability is exemplified for the antidepressant paroxetine. This substrate was selected, since it is transformed by demethylenylation to a catecholic metabolite and by

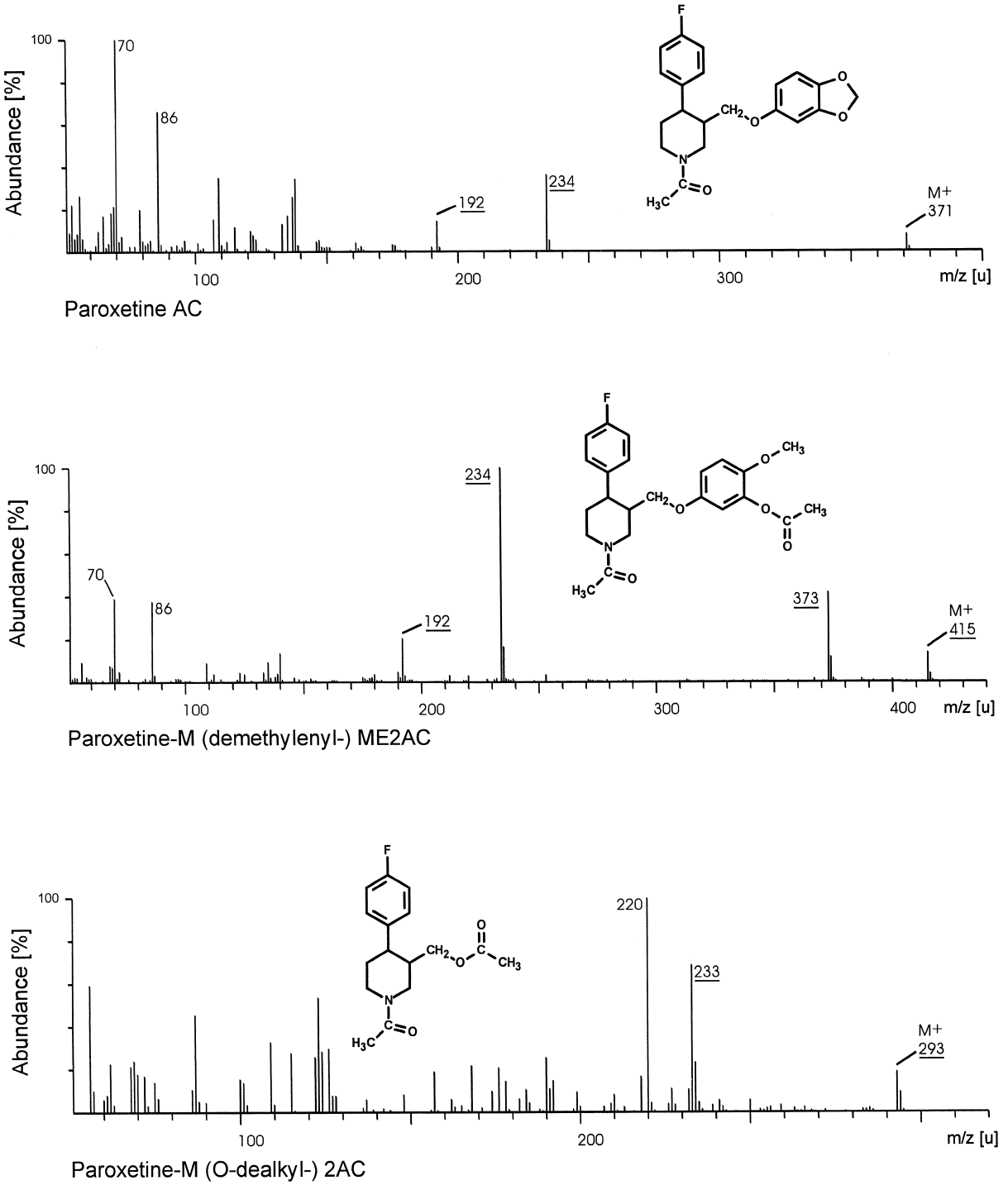


Fig. 6. Mass spectra and structures of acetylated paroxetine, of its demethylenylated metabolite (catechol) after (SAM) methylation and acetylation, and of its acetylated *O*-dealkyl metabolite. The underlined ions were selected for mass chromatography.

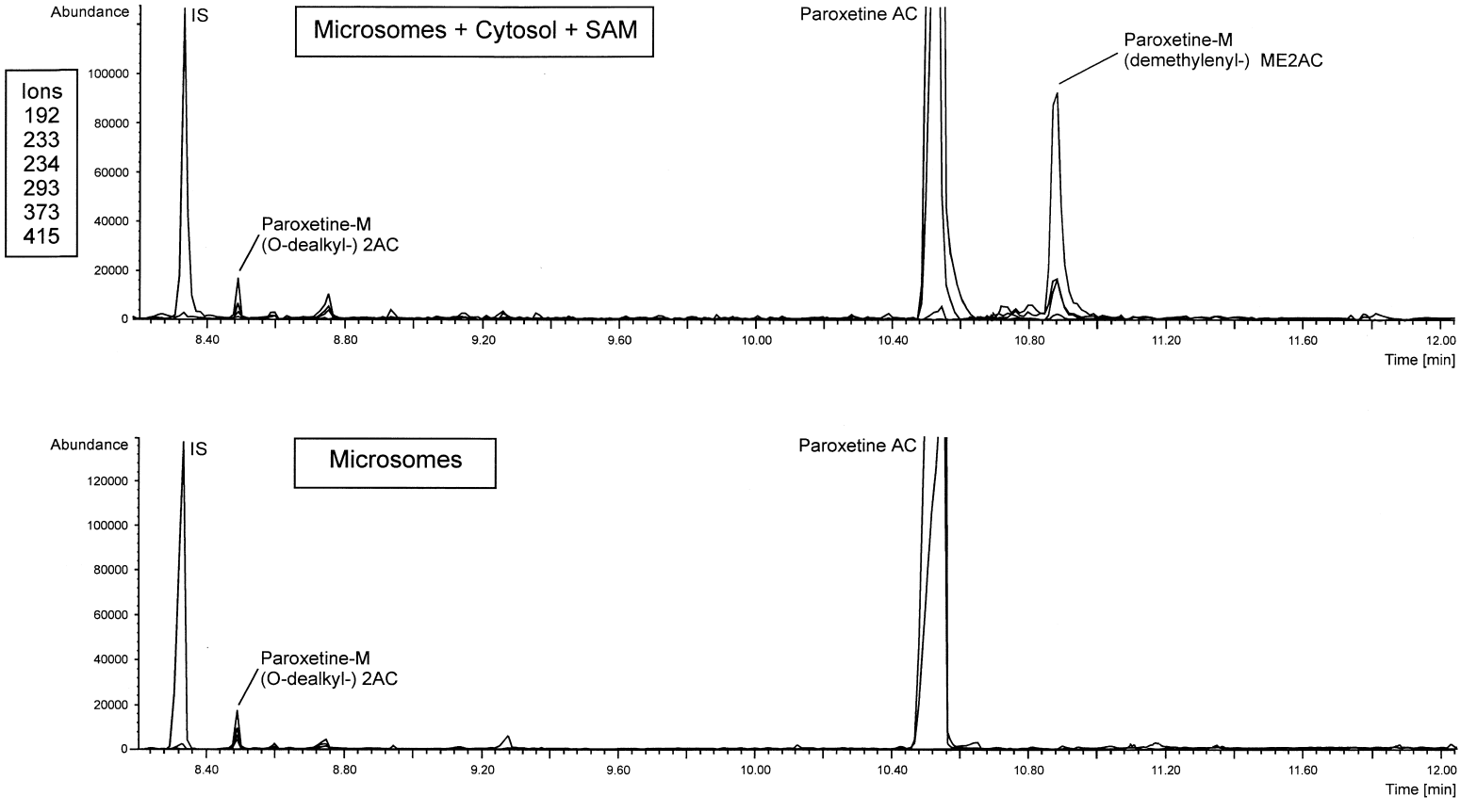


Fig. 7. Mass chromatograms of the ions m/z 192, 233, 234, 293, 373 and 415 selected from the mass spectra of paroxetine, its metabolites (Fig. 6) and of the I.S. methaqualone (Fig. 2). They were recorded in extracts of microsome preparations after incubation of paroxetine in presence of cytosol and SAM (upper part) and in absence of both (lower part).

O-dealkylation to a non-catecholic metabolite. Fig. 6 shows the mass spectra and structures of acetylated paroxetine, of its demethylenylated metabolite (catechol) after (SAM) methylation and acetylation, and of its acetylated *O*-dealkyl metabolite. From these mass spectra typical ions were selected for mass chromatography as shown in Fig. 7. These chromatograms were recorded in extracts of microsome preparations after incubation of paroxetine in presence of cytosol and SAM (upper part) and in absence of both (lower part). It can be seen that only in the sample with COMT (cytosol) and SAM, the (methylated and acetylated) catechol can be detected. The non-catecholic *O*-dealkyl metabolite was detected in both cases. The presence of the free (acetylated) catechol could be excluded, since its mass spectrum should also contain the diagnostic ions m/z 192 and 234. The differences in the retention times of acetylated catechols and the corresponding acetylated methoxy-hydroxy metabolites are typically less than 1 min [8,11,20,21]. In the meantime, using this procedure we have been able to study the influence of cytochrome P450 isoenzymes on demethylenation processes, e.g., of 3,4-methylenedioxy amphetamine designer drugs [4,22].

4. Conclusions

In conclusion, a universal GC–MS procedure was presented for the determination of catecholic metabolites in human and rat liver preparations after *in statu nascendi* derivatization using SAM catalyzed by cytosolic COMT. COMT activity determined using our assay should be at least 1 nmol product/min/mg protein. This could be confirmed by validated GC–MS quantification of 3-MHP after suitable incubation of the test substrate 3,4-DHP. In the meantime, the determination of catecholic metabolites has been proved in several in vitro metabolism studies.

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