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Potential of gas chromatography–atmospheric pressure chemical ionization–time-of-flight mass spectrometry for the determination of sterols in human plasma

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ABSTRACT

The application of Gas Chromatography (GC)–Atmospheric Pressure Chemical Ionization (APCI)–Time-of-Flight Mass Spectrometry (TOF-MS) is presented for sterol analysis in human plasma. A commercial APCI interface was modified to ensure a well-defined humidity which is essential for controlled ionization. In the first step, optimization regarding flow rates of auxiliary gases was performed by using a mixture of model analytes. Secondly, the qualitative and quantitative analysis of sterols including oxysterols, cholesterol precursors, and plant sterols as trimethylsilyl-derivatives was successfully carried out. The characteristics of APCI together with the very good mass accuracy of TOF-MS data enable the reliable identification of relevant sterols in complex matrices. Linear calibration lines and plausible results for healthy volunteers and patients could be obtained whereas all mass signals were extracted with an extraction width of 20 ppm from the full mass data set. One advantage of high mass accuracy can be seen in the fact that from one recorded run any search for m/z can be performed.

1. Introduction

Disorders in cholesterol biosynthesis can be responsible for severe health effects [1–3]. Furthermore, hyperabsorption of cholesterol or plant sterols by the intestinal mucosa are also discussed to be associated with the risk of cardiovascular diseases [4,5]. Most aberrations in cholesterol metabolism can be identified by the analysis of a sterol profile in plasma/serum. This should include not only cholesterol and its precursors, but also plant sterols such as sitosterol and campesterol, and cholesterol oxidation products. Cholesterol can be oxidized in vivo enzymatically and non-enzymatically. There is substantial evidence concerning the pathologic effects of oxysterols in atherogenesis, neurodegeneration, and inflammation. Comprehensive reviews of this topic have been published [6–10]. Several methods for sterol analysis from various biological matrices have been proposed, mostly based on gas chromatography (GC) [11–14], or liquid chromatography (LC) coupled to mass analyzers [15–17]. The GC based methods take advantage from excellent peak resolution which is especially important for sterol species which are very similar in chemical structure.

The enormous potential of high-resolution full spectrum acquisition techniques such as time-of-flight mass spectrometry (TOF-MS) in metabolomic and lipidomic research can be derived from the versatility of these instruments. The accurate mass data allow in a single run a wide-scope screening for unknown species and simultaneously a targeted-based approach depending on the aim of the analyst. Furthermore, full mass data sets, when they are once processed, allow any retrospective analysis in the future. These performance factors make TOF mass analyzers ideal for metabolomics, especially in combination with gas chromatography (GC).

Commonly, when using GC–MS the most prominent ionization mode is electron ionization (EI). As EI leads to strong fragmentation and does not always exhibit the molecular ion, softer ionization techniques are attractive to couple TOF-MS to GC. The first developments with atmospheric pressure chemical ionization (APCI) coupled to GC were carried out by Horning et al. in the 1970s [18,19]. 30 years later McEwen and McKay [20] and Schiewek et al. [21] demonstrated modifications of APCI sources to allow both LC–MS and GC–MS. Currently, there are commercially available sources from different manufacturers namely Waters Corporation, Bruker Daltonics and Agilent Technologies. However, until now GC–APCI–TOF-MS has not widely spread. Advantageous applications have been shown in pharmaceutical research [22], pesticide residue analysis [23,24], and metabolic profiling [25–27].

In APCI the influence of water is crucial because ionization is caused primarily by ion-molecule proton transfer reactions between protonated water clusters and analyte molecules. As pointed out by several authors [22,27] constant humidity level in the APCI source is crucial to establish consistent ionization.

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In the present paper we demonstrate a modification of a commercial APCI source to control humidity and corresponding ionization conditions in which the modified system is applied to determinations of sterols in human plasma.

2. Materials and methods

2.1. Chemicals and solutions

Hexane, methanol, sodium chloride, and potassium hydroxide were purchased from VWR GmbH (Darmstadt, Germany). N-methyl-N-trimethylsilyl-trifluoracetamide (MSTFA) was obtained from Macherey-Nagel GmbH & Co. KG (Düren, Germany). 7β-, 24(S)- and 27-hydroxycholesterol, 7-ketocholesterol, and lanosterol were purchased from Avanti Polar Lipids (Alabaster, Alabama, USA). Benzophenone, methyl stearate, methyl palmitate, campesterol, sitosterol, lathosterol, campesterol D6, sitosterol D6, and butylated hydroxytoluene were obtained from Sigma–Aldrich Chemie GmbH (Taufkirchen, Germany). 7-Ketocholesterol D7 was purchased from Toronto Research Chemicals (North York, Ontario, Canada) and 27-hydroxycholesterol D6 from Sugaris GmbH (Muenster, Germany).

Benzophenone, methyl stearate, methyl palmitate, and cocaine hydrochloride were dissolved in methanol/methylene chloride as stock solutions. For the preparation of a working solution these stock solutions were mixed and further diluted in methanol to a concentration of 100 μg/ml.

2.2. Human plasma samples and sample preparation

EDTA-plasma was obtained from healthy volunteers and a cerebrotendinous xanthomatosis (CTX) patient by standard venipuncture techniques. Samples were immediately centrifuged at 2000*g* for 10 min. Butylated hydroxytoluene was added to all plasma samples at a concentration of 50 μg/ml. Plasma samples were stored in aliquots at −80 °C.

The sample preparation method was based on a method by Dzeletovic et al. [11] with some modifications [14]. To 400 μl sample 40 μl of a mixture of deuterium-labeled internal standards (5 μg/ml 27-hydroxycholesterol D6, 25 μg/ml 7-ketocholesterol D7, 10 μg/ml campesterol D6, and 10 μg/ml sitosterol D6 in methanol) was added. To cleave ester bondings, alkaline hydrolysis was performed with 2 ml freshly prepared 1 M sodium hydroxide in ethanol (5.61 g in 100 ml) for 60 min at 25 °C under continuous agitation. Afterwards, the reaction solution was adjusted to pH 7 with phosphoric acid and 2 ml sodium chloride solution. Sterols were extracted with 2 × 3 and 1 × 1 ml n-hexane. The solvent was evaporated to dryness under reduced pressure. The residue was dissolved in 50 μl of the reagent MSTFA for trimethylsilylation. Derivatization reaction was performed for 60 min at 60 °C. The derivatized samples were 1:10 diluted with hexane and transferred to GC vials for direct injection.

2.3. GC–APCI–TOF-MS

Gas chromatography was performed on an Agilent 7890A gas chromatograph equipped with a multimode inlet, a HP-5 ms column (30 m, 0.25 mm, 0.25 μm df). The carrier gas was helium with a constant flow of 1.2 ml/min. 1 μl of the model analytes was injected with a split ratio of 1:100. The derivatized sterol samples were injected in splitless mode.

A GC–APCI interface from Agilent was used. The APCI design implemented a makeup gas addition port inside the GC oven to accommodate the delivery of a makeup gas for assistance in ionization. Helium was used as makeup gas. Water vapor was introduced by connecting a pressure controlled reagent bottle of N_2O or synthetic air via a water-filled flask directly into the APCI interface applying different pressure adjustments. The transfer line between the GC and the APCI interface was heated to 300 °C. MS analysis was performed using a 6540 QTOF (Agilent Technologies, Waldbronn, Germany). The corona discharge needle was operated at +1000 nA. Positive ion mass spectra were acquired in a mass range from 100–1000 m/z with a scan rate of 8 spectra/s. Mass calibration was done daily, based on a special tuning mix (Agilent). Mass accuracies were better than $\Delta m = 3$ ppm. Data analysis was performed using Mass Hunter Software (Agilent).

3. Results

3.1. Optimization of the APCI parameters

The Agilent GC–APCI interface is designed with a short, heated transfer tube to connect the GC capillary to the APCI source and to avoid cold spots. The outlet of the GC column and the inlet to the MS are at atmospheric pressure, allowing as much as five times higher column gas flow than dedicated GC systems. The interface has a small opening to the lab to allow for trace levels of water from the atmosphere to enter into the source. In principle, these trace levels of water vapor are sufficient for ionization. H_3O^+ and protonated water clusters $(H_2O)_nH^+$ are formed which provide further ionization of molecules with higher proton affinity by ion-molecule proton transfer reactions between protonated water clusters and the analyte molecules.

In the first experimental setup, MS intensities of a mixture of model compounds with different structure and polarity were studied. These model analytes were benzophenone (m/z $[MH]^+$ 183.0789), methyl palmitate (m/z $[MH]^+$ 271.2611), methyl stearate (m/z $[MH]^+$ 299.2923), and cocaine (m/z $[MH]^+$ 304.1522). A GC oven program was chosen to cover a retention time window of five minutes. During several runs on consecutive days, signal intensities varied dramatically about two orders of magnitude due to fluctuations of temperature and humidity in the lab. Therefore, we installed a polytetrafluoroethylene tubing leading N_2O or synthetic air from a reagent bottle via a water flask equipped with a porous frit into the APCI interface to ensure consistent humidity inside the source. The humidity could be controlled via pressure regulation of the reagent bottle and was monitored by means of a humidity sensor which was implemented into the APCI source. To determine the optimal humidity for ionization signal intensities of the model analytes were recorded at different humidity values. Fig. 1 shows the dependence of signal intensity on humidity. Every run was threefold repeated to check on reproducibility. It is obvious from Fig. 1 that ionization is enhanced up to 1.5–1.8% humidity followed by a significant drop with increasing water content. On the other hand, there was poor precision of the signal intensities with higher water content as indicated by the large error bars. This might be attributed to stronger turbulences inside the source. Henceforward, a compromise between good ionization conditions and signal stability had to be found.

Higher intensities could be observed when the ion source region (the immediate vicinity of the MS capillary) is purged with dry nitrogen. The dry gas reduces the amount of contaminants and excess water vapor so that ionization is primarily by H_3O^+ proton transfer. Fig. 2 shows the dependence of the signals on the dry gas flow rate. The highest intensities could be achieved with the highest dry gas flow rate. Additionally, we show the dramatic decrease of the ion $(H_2O)_2H^+$ (m/z 37.0290) to demonstrate the effect of dry gas on water clustering. At 5 L/min this ion was no longer detectable.

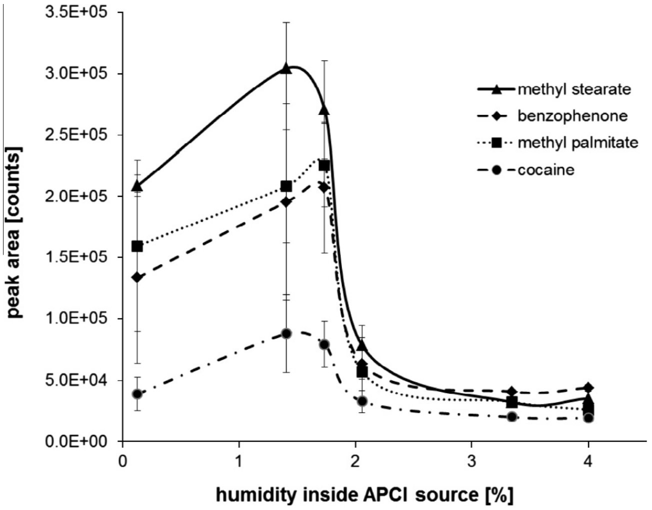

Fig. 1. Influence of humidity on signal intensities of model compounds. Concentration of model analytes 100 µg/ml. Split ratio 1:100. Error bars indicate standard deviation of three repetitive injections.

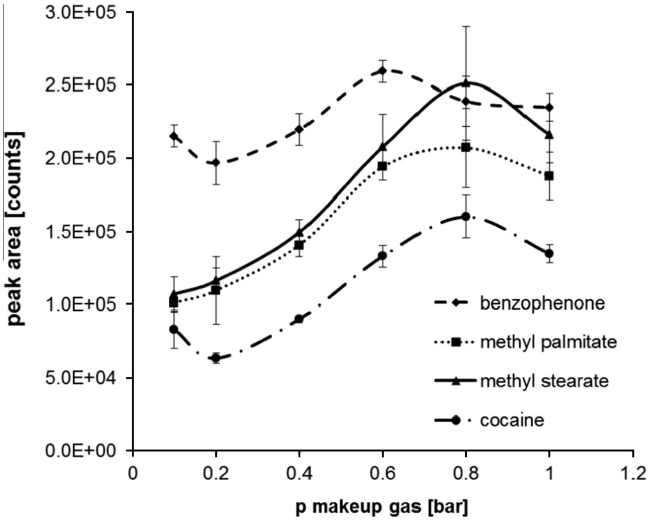

Fig. 3. Influence of makeup gas on signal intensities of model compounds. Experimental conditions see Fig. 1.

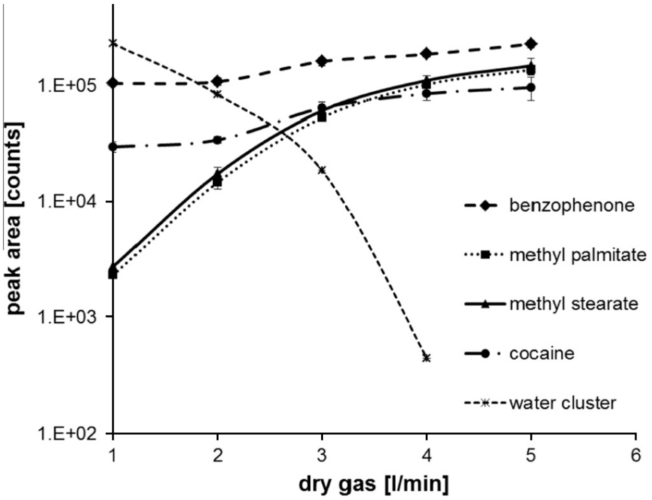

Fig. 2. Influence of dry gas flow on signal intensities of model compounds. Experimental conditions see Fig. 1.

The third influence on this experimental setup was seen in variation of the makeup gas. The makeup gas assists in ionization ensuring high flow rate compared to standard GC efflux. The makeup gas was pressure controlled. However, as seen in Fig. 3 there is an optimum for efficient ionization at around 0.6 bar makeup gas helium. The use of N_2O as makeup gas gave comparable results. Providing a higher pressure, the precision became worse. This behavior is difficult to explain. One explanation for the increasing imprecision could be seen in more turbulent flow and an uncontrolled temperature gradient inside the transfer line.

3.2. Application to sterol analysis

A standard mix of sterols was prepared by addition of different concentrations of 7β-, 24(S)- and 27-hydroxycholesterol, 7-ketocholesterol, lanosterol, lathosterol, campesterol, and sitosterol to diluted EDTA-plasma. After complete sample preparation the TMS derivatives were analyzed by GC–APCI–TOF-MS with the optimized source conditions, i.e. humidity corresponding to 0.3 bar N_2O, dry gas 5 L/min and makeup gas 0.6 bar.

For the sterols under investigation the correct masses were calculated according to $[M+H]^+$, $[M-TMS+H]^+$, and $[M-OTMS+H]^+$ whereas M corresponds to the trimethylsilyl derivative of the sterol species. Retention times were found by injecting individual substances. The performance of GC–APCI–TOFMS was evaluated with a calibration study covering the endogenous range in human plasma. Each calibrator was measured three times over a two day period. In Table 1 the calculated m/z values, the measured m/z values, linear ranges, and the inter-assay coefficients of variation are given for the sterol species. The most intense mass signals were chosen for each component as quantification signal except for 27-hydroxycholesterol, 7β-hydroxycholesterol, and lathosterol where we used the mass signal corresponding to $[M-OTMS+H]^+$ or $[M+H]^+$, respectively. The mass signals were extracted with an extraction width of 20 ppm from the full mass data set.

Although coelution was observed for sitosterol and lanosterol mass spectral peak deconvolution enabled the discrimination of these compounds. For calibration, 27-hydroxycholesterol, 7-ketocholesterol, campesterol, and sitosterol were normalized by the corresponding stable isotope-labeled standard. Lanosterol, lathosterol, and 24(S)-hydroxycholesterol were normalized by the closest eluting internal standard (see Table 2).

To verify the method's accuracy plasma samples of a healthy volunteer and of a patient with cerebrotendinous xanthomatosis (CTX) were prepared according to the sample preparation protocol. The samples were analyzed and the concentrations calculated. Fig. 4 shows a typical chromatogram of a healthy volunteer which exhibits the extracted masses of lathosterol, 7β-hydroxycholesterol, campesterol, lanosterol, sitosterol, 24(S)-hydroxycholesterol, 7-ketocholesterol, and 27-hydroxycholesterol. The mass extraction leads to well-resolved and symmetric peaks despite the fact that the extraction width of 20 ppm is rather broad.

The concentrations of sterols were consistent with the literature and to own experience [11,14]. It is noteworthy that in CTX no 27-hydroxycholesterol can be formed due to a missing enzyme (CYP27A1) in the bile acid synthesis pathway [28,29]. Therefore, screening for this metabolite can be easily performed from any processed TOF data set. Furthermore, this plasma sample was stored for more than three months and autoxidation of plasma cholesterol might lead to an artificial elevated value of 7-ketocholesterol and 7β-hydroxycholesterol.

Table 1
Analytical characteristics of sterol determinations by GC–APCI–QTOF.

Compound	m/z calculated	m/z measured	Δm/z in ppm	Linear range (ng/ml)	*R*	Inter-assay CV at concentration*
7β-Hydroxycholesterol	457.3860	457.3851	2.0	5–100	0.998	10.7% at 17 ng/ml
27-Hydroxycholesterol	457.3860	457.3845	3.3	10–210	0.995	12.8% at 33.3 ng/ml
24(S)-Hydroxycholesterol	367.3364	367.3352	3.2	10–152	0.998	15.6% at 24 ng/ml
7-Ketocholesterol	473.3809	473.3798	2.3	5–120	0.993	n.a.
Lanosterol	409.3829	409.3819	2.4	5–516	0.995	2.9% at 78 ng/ml
Lathosterol	459.4017	459.4006	2.4	200–4050	0.997	4.6% at 645 ng/ml
Sitosterol	397.3829	397.3822	1.8	300–5000	0.993	7.1% at 798 ng/ml
Campesterol	383.3672	383.3679	1.8	300–5000	0.999	8.1% at 784 ng/ml

n.a. not analyzed.
* Obtained from calibrator (diluted plasma, spiked with standards), *n* = 3.

Table 2
Concentrations of sterol species in EDTA plasma obtained by GC–APCI–QTOF in ng/ml.

Sample	Lanosterol	Lathosterol	Campesterol	Sitosterol	7β-OH-cholesterol	24(S)-OH-cholesterol	7-Ketocholesterol	27-OH-cholesterol
Healthy volunteer	121.0	1730.9	4508.3	1815.5	14.2	58.9	31.3	120.7
CTX patient	108.2	1367.5	1308.8	761.6	27.5	39.3	244.6	n.d.

n.d. not detectable.

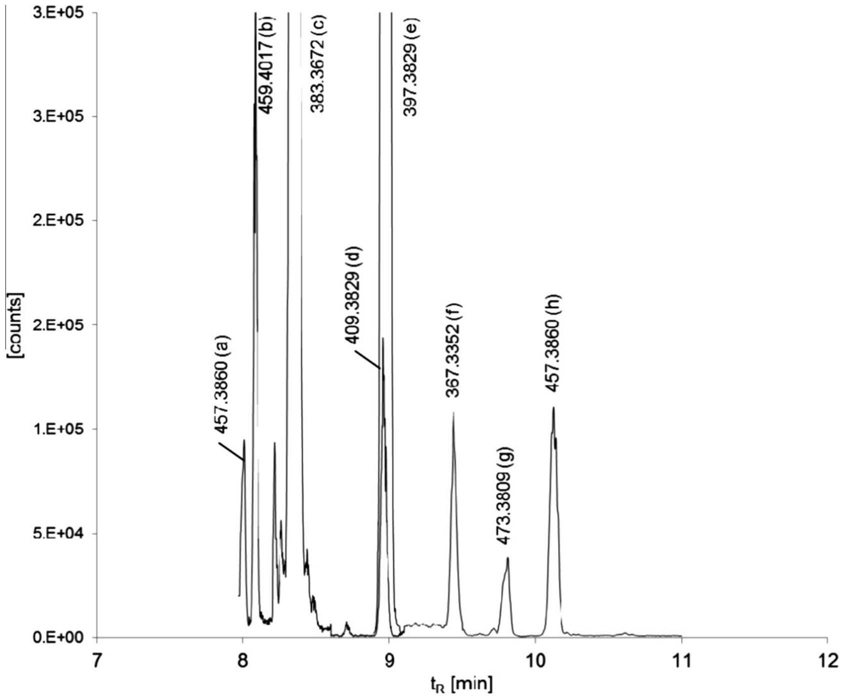

Fig. 4. Extracted ion GC–APCI–TOF-MS chromatogram of an EDTA plasma sample of a healthy volunteer after trimethylsilyl derivatization. (A) 7β-hydroxycholesterol, (B) lathosterol, (C) campesterol, (D) lanosterol, (E) sitosterol, (F) 24(S)-hydroxycholesterol, (G) 7-ketocholesterol and (H) 27-hydroxycholesterol.

4. Discussion

In this study we applied GC–APCI–TOF-MS after modification and optimization of a commercial APCI interface to the quantitative analysis of sterols including oxysterols, cholesterol precursors, and plant sterols. Linear calibration lines were obtained for several sterol species. Linearity and precision could depend on turbulences inside the APCI source resulting from the makeup gas and the assisting water vapor gas pressure. A critical factor for uniform and reproducible ionization conditions is well-controlled humidity. This is established by our experimental setup. However, turbulent disturbances can cause more noise. Therefore, deuterated standards for the analytes of interest should be used to compensate flow instabilities.

The main advantage of high mass accuracy can be seen in the fact that from one recorded run any search for m/z can be performed. For example, dihydrolanosterol is also a precursor in cholesterol biosynthesis, formed by 24-dehydrocholesterol reductase (DHCR24) from lanosterol. The $[M\text{-}OTMS+H]^+$ ion of the TMS derivative is calculated to 411.3984. This mass signal was found both in the sample of the CTX patient and of the healthy volunteer, extracted with an extraction width of 10 ppm and verified by the retention time of the standard substance.

In conclusion, the advantages which are known for coupling LC to MS can be applied to GC–TOF-MS such as accurate mass measurement as well as reaction ion monitoring because the instrument used by us was a hybrid quadrupole TOF-MS, i.e. equipped with a quadrupole and a collision cell. However, the use of a precursor filter (Q1) followed by collision and product ion generation did not enhance the sensitivity of the analysis of sterols compared to accurate mass determination of the precursor ion alone. The potential of coupling GC with its extraordinary separation performance, APCI as a soft ionization technique and the high mass accuracy of TOF-MS is attractive for the identification of unknown and unexpected components.

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