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Development of a high-performance liquid chromatographic–atmospheric pressure chemical ionization–tandem mass spectrometric assay for β-tigogenin cellobioside in human serum

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Abstract

A specific high-performance liquid chromatographic–atmospheric pressure chemical ionization tandem mass spectrometric assay for the quantitative determination of β-tigogenin cellobioside in human serum is described. Serum cleanup and acetylation of the analyte were required to achieve the desired lower limit of quantification, 10 ng/ml. The precision of the assay was better than 13% over a serum concentration range of 10–500 ng/ml.

Keywords: β-Tigogenin cellobioside

1. Introduction

β-Tigogenin cellobioside (TC; Fig. 1) is a novel cholesterol absorption inhibitor currently under evaluation [1,2]. An analytical method for its quantitative determination in human serum was needed to support clinical studies. Preliminary pharmacological data suggested that a lower limit of quantification of 10 ng/ml would be required. Even though this level was not particularly low, the lack of a significant chromophore, fluorophore or electrophore led us to consider mass spectrometric detection.

Atmospheric pressure ionization mass spectrometry with either chemical ionization (APCI-MS) or pneumatically assisted electrospray (ES-MS) has allowed mass spectrometric analysis of complex

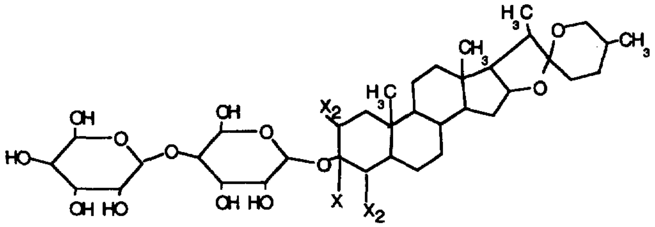

Fig. 1. Structures of β-tigogenin cellobioside and its pentadeuterated internal standard.

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molecules such as proteins [3] and ionophores [4]. Its combination with high-performance liquid chromatography (HPLC) is particularly applicable to the quantitative analysis of drugs in biological fluids down to 10 pg/ml [5–7]. The direct analysis of TC by HPLC–APCI-MS or HPLC–ES-MS was not successful. This communication discusses the successful development of an assay requiring sample cleanup and analyte derivatization, followed by HPLC–APCI-MS–MS.

2. Experimental

2.1. Materials

TC and a pentadeuterated internal standard (I.S.) were synthesized in-house [2]. The isotopic distribution of the I.S. was $^2H_0$, <0.4%; $^2H_1$, 1%; $^2H_2$, 1%; $^2H_3$, 2%; $^2H_4$, 1%; and $^2H_5$ 100%. Stock solutions of TC and I.S. were made up at 100 μg/ml in methanol and diluted in methanol as needed and were stored at 4°C when not in use. All solvents were HPLC grade. Reagent grade acetyl chloride (Fisher) was used as received. All glassware was silanized in a vacuum oven following the procedure of Fenimore et al. [8].

2.2. Sample preparation

To clean 15-ml centrifuge tubes was added 1.0 ml of a human serum sample followed by 50 μl of a 100 μg/ml (total 5 μg) of the I.S.. The solution was vortex mixed for 5 s, 5 ml of hexane was added and the tube vortexed for an additional 10 s. The tubes were centrifuged for 3 min at 1500 *g* and the hexane layer was removed by aspiration. During the removal of the hexane layer, any emulsion present at the interface was left behind. To the cleaned serum was added 2 ml of acetonitrile and the tubes vortexed for 10 min on a multi-tube vortexer. After centrifugation at 2500 *g* for 10 min, the supernatant was transferred to clean tubes and evaporated to dryness on a vortex evaporator set at 50°C.

The residue was suspended in 100 μl of acetonitrile by vortexing for 5 s, sonicating for 1 min and vortexing for an additional 10 min. Acetyl chloride (200 μl) was added to each tube and the resulting solution vortexed for 60 s. The samples were capped and allowed to sit overnight at room temperature. Excess reagent and solvent were removed by vortex evaporation (50°C) and the residue dissolved in 500 μl acetonitrile by vortexing for 5 s, sonicating for 1 min and vortexing for an additional 10 min. The tubes were centrifuged for 3 min at 2500 *g*, the supernatant transferred to clean centrifuge tubes and the acetonitrile removed by vortex evaporation (50°C). Derivatized drug and I.S. were reconstituted in 60 μl of mobile phase and transferred to the HPLC autosampler vials.

2.3. HPLC–MS analysis

Analyses were performed on a SCIEX (Thornhill, Canada) API III triple quadrupole mass spectrometer. Two chromatographic systems were utilized. Initial electrospray ionization studies were carried out on a 1×150 mm BDS Hypersil C_{18} column (Keystone, Bellafonte, PA, USA) using a mobile phase of acetonitrile–tetrahydrofuran–10 m*M* ammonium acetate (70:20:10). A dual piston syringe pump (ABI Biosystems, Foster City, CA, USA) supplied 40 μl/min of the mobile phase to the 1 mm column. The chromatographic system utilized for APCI studies was a 3.9×150 mm NovaPak C_{18} column (Waters, Milford, MA, USA) using a mobile phase of methanol–2-propanol–water (50:40:10) degassed through a 0.7-μm glass fiber filter. A Constametric 3000 pump delivered the mobile phase at 1 ml/min. Both analytical columns were preceded by a 5 μm precolumn stainless steel filter (Alltech, Deerfield, IL, USA). A Perkin-Elmer (Norwalk, CT, USA) ISS 100 autosampler injected 50-μl sample aliquots onto the column at 2.7 min intervals. Analyte and I.S. responses were collected from 1.5 to 2.5 min after sample injection. Under these HPLC conditions, TC and I.S. coeluted at 1.9 min.

The HPLC flow was introduced into the mass spectrometer with either a pneumatically assisted ES ion source or an APCI ion source. The ES system was operated at 5000 V and nitrogen nebulizing gas at 60 psi. The APCI system consisted of a heated nebulizer interface (nebulizer probe temp=450°C) combined with corona discharge (5.0 μA). Positive or negative ions formed by either interface were sampled into the quadrupole mass filter via a 0.0045″ sampling orifice. The instrument was operated under unit mass resolution. Collision induced dissociation (CID) was performed using argon at 5.50×10^{14} atoms/cm^2 and a laboratory frame collision energy of 18 eV. For quantitative analysis, drug and I.S. were measured using multiple reaction monitoring (MRM): i.e. monitoring selected CID product ions of specific parent ions. Protonated molecule ions for acetylated drug and acetylated I.S. at *m/z* 1035 and 1040, respectively, were dissociated to produce a common product ion at *m/z* 331.

2.4. Quantitation

Control serum was fortified with TC at seven concentrations in duplicate over the range of 10 to 500 ng/ml. Standard curves were constructed to compare analyte concentration vs. peak-area ratios of drug over I.S.. A linear regression model, with $1/x^2$ weighting, was used to generate the standard curves.

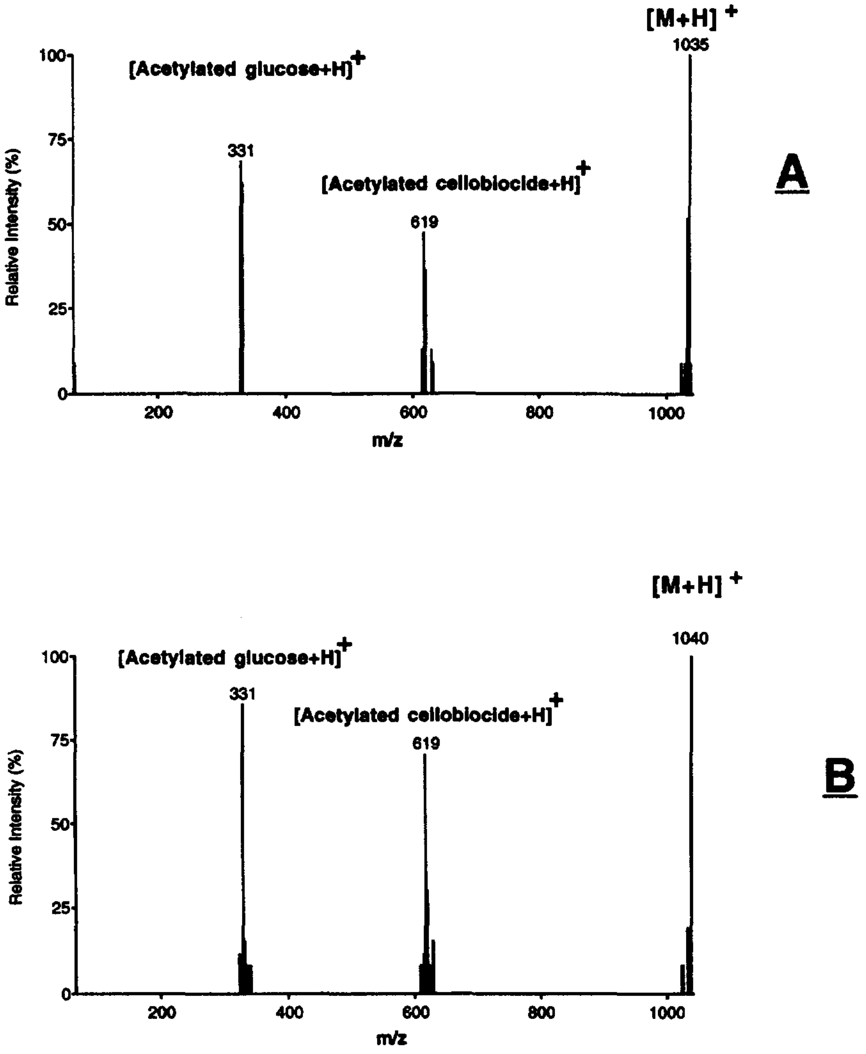

Fig. 2. CID product ion spectra of fully acetylated β-tigogenin cellobioside (A) and D_5-β-tigogenin cellobioside (B).

3. Results and discussion

The positive ion spectrum of TC obtained with the heated nebulizer APCI interface showed three abundant ions representing the protonated molecule at m/z 741, the loss of one sugar at m/z 579 and the loss of two sugar units at m/z 471. This reflects thermal breakdown of TC in the heated nebulizer interface. Pyrolysis of thermally labile compounds in the heated nebulizer interface has previously been noted [9]. This facile loss of the sugar moieties limits the sensitivity in APCI-MS. Attempts to reduce this degradation by lowering the temperature of the interface were not successful.

Under ES ionization conditions and in the presence of ammonium acetate, TC forms abundant ammonium adducts in positive ion mode or acetate adduction ions in the negative ion mode. Higher parent ion abundance was obtained in the negative ion mode. A single product ion representing the loss

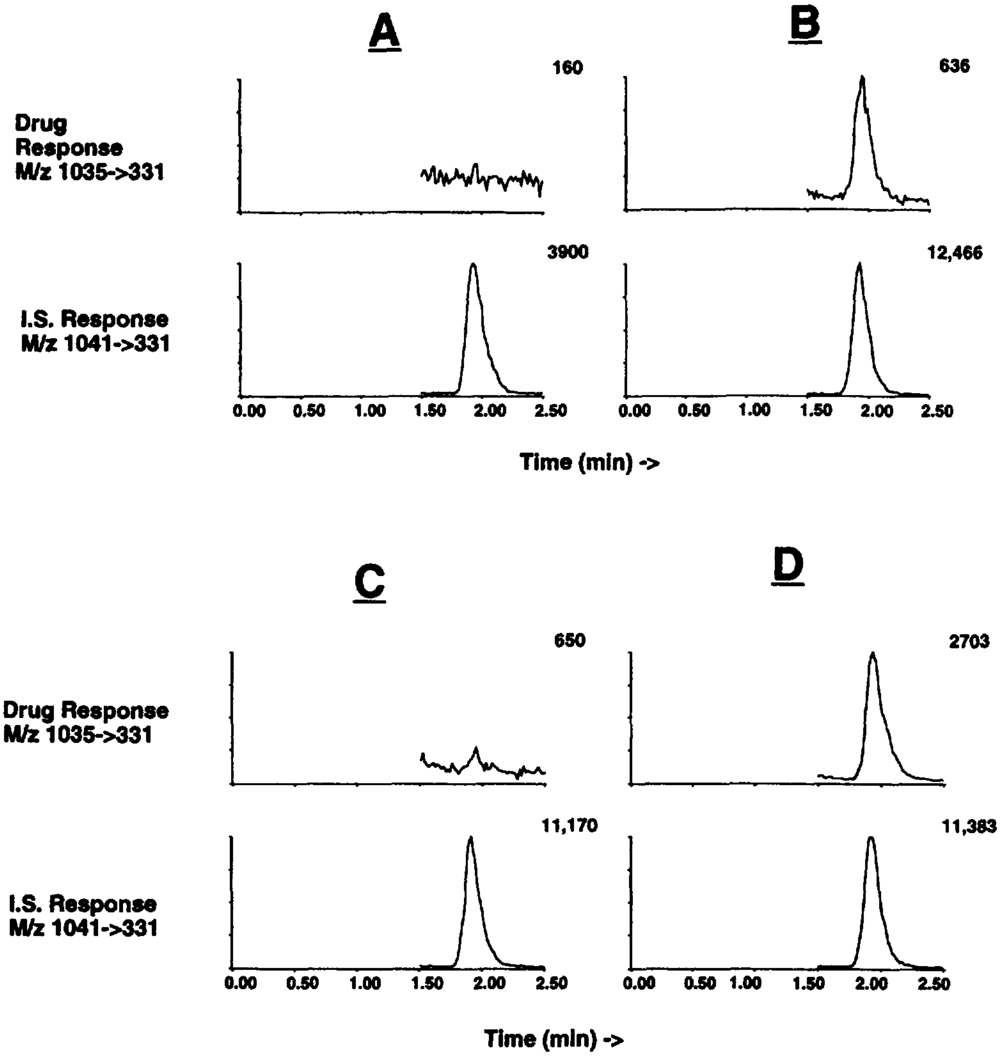

Fig. 3. Representative HPLC–APCI-MS MRM chromatograms from analysis of (A) blank human serum, (B) blank human serum fortified with β-tigogenin cellobioside at 10 ng/ml, and from human serum dosed with 3 g β-tigogenin cellobioside (C) pre-dose and (D) 2 h post-dose (assayed level 51 ng/ml).

of the acetate moiety was obtained by CID of the acetate adduct. The MRM transition *m/z* 800→741 produced an adequate response for 500 pg TC. However, after several injections, TC response steadily decreased. The response could be regenerated by replacing the fused-silica transfer line of the ionspray interface. This effect was probably due to adsorption of the surface active TC. The surface activity of TC was also evident in significant and irreproducible losses during initial sample preparation steps. To reduce the surface activity of TC and to improve its recovery during sample preparation and analysis, derivatization was investigated.

Derivatization of the seven hydroxyls of TC to acetates, although resulting in lower ES response, contributed to a significant reduction of adsorptive losses. As expected, the derivatized drug exhibited improved thermal stability in the heated nebulizer interface, promising successful analysis by APCI. The overall recoveries for drug and I.S. using this procedure were 49% and 57% respectively.

The CID product ion spectra of the derivatized drug and derivatized I.S. are shown in Fig. 2. The APCI generated protonated molecule ions at *m/z* 1035 and 1040 produce an acetylated cellobioside product ion at *m/z* 619 and an acetylated glucose product ion at *m/z* 331. Since the latter was the most intense product ion, it was used for subsequent MRM analyses. The signals for drug and I.S. were free of interferences from matrix components. Typical responses for drug and I.S. from fortified control serum samples containing 0 and 10 ng/ml of TC are shown in Fig. 3(A and B). The assay was used to determine TC levels in serum from human subjects in a clinical study. The data for pre-dose and 2 h post-dose serum samples is shown in Fig. 3(C and D).

Table 1
Intra-assay precision and accuracy: analysis of human serum samples containing known amounts of β-tigogenin cellobioside; six replicates at each concentration

Concentration added (ng/ml)	Concentration found (mean±S.D.) (ng/ml)	Accuracy (%)	Precision (% C.V.)
10	10.1±0.61	101	6
20	18.8±2.4	94	13
40	42±2.9	105	7
60	61±4.3	101	7
100	104±3.1	104	3
250	249±7.5	100	3
500	495±20	99	4

Table 2
Inter-assay accuracy and precision: analysis of human serum samples containg known amounts of β-tigogenin cellbioside: two replicates at each concentration on sixteen different days

Concentration added (ng/ml)	Concentration found (mean±S.D.) (ng/ml)	Accuracy (%)	Precision (% C.V.)
25	26±2.1	102	8
100	103±7.2	103	7
400	404±24	101	6

Table 13 lists the accuracy and precision of the assay over the concentration range of 10–500 ng per ml of serum. The measured concentrations were within 6% of their theoretical values. Intra assay precision for six replicates at different concentrations was 13% or better. Inter assay precision and accuracy data determined over several weeks were also acceptable (Table 2).

4. Conclusions

Despite the profound success and wide applicability of direct HPLC–APCI-MS for quantitative analysis, ancillary analytical techniques, such as derivatization, are sometimes required. The current assay benefits from derivatization for three reasons. First, the surface activity of the drug is greatly reduced, eliminating adsorptive losses. Second, since background interferences in APCI-MS increase with decreasing mass, derivatization to a higher molecular weight species decreases chemical noise resulting in a corresponding increase in signal to noise. Third, thermal degradation of the analyte is reduced. For TC, the performance of the assay improved sufficiently to allow the successful determination of the drug at clinically relevant concentrations.

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