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Journal of Chromatography A, 871 (2000) 173–180

JOURNAL OF CHROMATOGRAPHY A

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Enantiomeric separation of clenbuterol by transient isotachophoresis–capillary zone electrophoresis–UV detection New optimization technique for transient isotachophoresis

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Abstract

A method for the in-line preconcentration and enantioseparation of clenbuterol by transient isotachophoresis–capillary zone electrophoresis–UV absorbance detection (transient ITP–CZE–UV) has been developed. It implies the use of dimethyl-β-cyclodextrin as chiral selector and the application of a hydrodynamic counterflow during the ITP step. ITP is used to focus the sample constituents prior to CE whereas a counterpressure counterbalances the electrophoretic migration of the compounds. The sample is then focused and kept stationary in the proximity of the capillary inlet before CZE separation, leading to an extended-volume ITP–CZE system. A new strategy for the fast optimization of the counterpressure has been developed which implies the measurement of the hydrodynamic and electrophoretic velocities of the analyte during ITP. The in-line preconcentration and enantioseparation of clenbuterol selected as model compound was optimized using this method. Salbutamol was chosen as internal reference in order to check the reproducibility of the method. A 173-nl volume of aqueous sample solution was injected which implies an improvement of the injection volume of about 16 and a resolution of 4.8 was obtained for the clenbuterol enantiomers. A concentration detection limit of 10^{-6} mol/l was readily achieved for clenbuterol and salbutamol using only 3 min ITP preconcentration in in-line counterflow transient ITP–CZE–UV. Thanks to its fast optimization, the method is applicable to any enantioseparation by means of only five very short preliminary measurements.

Keywords: Isotachophoresis–capillary zone electrophoresis; Optimization; Enantiomer separation; Clenbuterol

1. Introduction

Many chemical compounds used in pharmaceutical formulations are chiral [1]. For several drugs, one of the two enantiomers possesses a different pharmacological activity or can even be toxic implying a demand for analytical methods with high resolution power and high efficiency. Compared to other analytical techniques used in chiral analysis such as high-performance liquid chromatography (HPLC), capillary zone electrophoresis (CZE) can offer several advantages including high separation efficiency, extremely small injection volumes of the sample, speed of method development and low reagent costs [2,3]. Various chiral selectors are described in the literature; among them, the most widely used cyclodextrins [4–7]. However, the major drawback with CZE is the limited sample concentration sensitivity due to the restricted injection

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0021-9673/00/$ – see front matter
PII: S0021-9673(99)00983-8

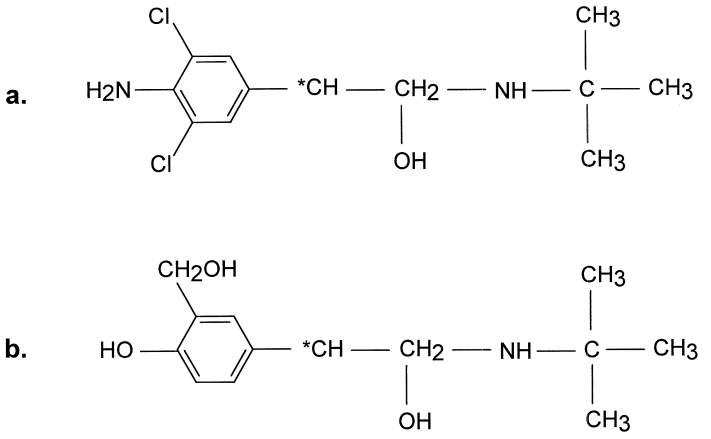

Fig. 1. Chemical structures of clenbuterol, M_r 276.1 (a); salbutamol, M_r 239.2 (b).

volume (typical injection volumes in CZE are in the low nanoliter range) and to the short optical path length, available for UV absorbance detection. To overcome those problems, several techniques have already been mentioned in literature involving chromatographic or isotachophoretic methods for sample concentration prior to CZE [8].

Isotachophoresis (ITP) has been demonstrated to be a very powerful sample enrichment technique in on-line coupling with CZE [9–12]. More specifically, when the ion of the selected CZE buffer has a higher electrophoretic mobility than the sample ions, the transient ITP technique allows ITP concentration and CZE separation to be achieved subsequently in a single capillary [13,14].

The use of a counterflow during ITP, keeping the focused sample stationary in the proximity of the capillary inlet prior to CZE, has often been employed to enhance the sample loadability of the system and improve the CZE separation path length. A counterflow can be achieved by using a difference in height [13], a T-junction which connects the capillary to a vial containing CZE buffer [15], the electroosmotic flow [16,17] or a counterpressure [10,18–20]. Counterpressure is particularly interesting for the reason that it can be automatically performed by commercial equipments which enable pressure fine tuning. Owing to the self-correction of diffusion band broadening during ITP, no loss of efficiency is observed when a counterflow is generated in the capillary. The counterflow is commonly optimized by addition of crystal violet to the sample and visualization of the concentrated colored zone through the wall of the capillary after removing of the polyimide coating [21,22]. However, a capillary with minimal 100 μm inner diameter has to be employed to be able to visualize the concentrated zone. In addition, a capillary which polyimide coating has been removed must be handled with a lot of care to prevent it from breaking. Another approach based on the current monitoring during ITP was developed in order to determine the suitable counterpressure that has to be applied in counterflow ITP [15,23]. The counterpressure was applied until 95% of the maximum current value observed when the sample was focused at the capillary inlet and almost no terminating electrolyte was left in the capillary. This method is highly reliable although time consuming in experiments because the maximum current is depending on the conductivity at the end of ITP, thus depending on the volume and concentration of the injected sample.

The method presented in this paper implies another strategy for counterflow transient ITP optimization based on the measurements of the electrophoretic and hydrodynamic migration rates of the analytes during ITP–CZE. Taking into account those observed values, it was possible to predict the migration of the sample in the capillary during ITP and to apply the counterpressure which was required to keep the sample stationary at the capillary inlet prior to the CZE separation. Clenbuterol was select-

ed as the model compound for the method development. Therefore, the demand for the analytical drug residue monitoring of clenbuterol is now increasing. The developed method benefits from the in-line combination of ITP and CZE for chiral separation. Clenbuterol enantiomers were preconcentrated and completely resolved in an extended-volume ITP–CZE system. The use of a counterflow during ITP did not disturb the enantioseparation. The optimization of counterflow ITP was fast and did not necessitate any tedious capillary handling. Moreover it allowed the use of a narrow-bore capillary, 50 μm I.D., thus providing a higher efficiency for enantioseparation.

2. Experimental

2.1. Chemicals and reagents

All chemicals used were of analytical grade. Aqueous solutions were prepared using water purified with a Milli-Q system (Millipore, Bedford, MA, USA). Ammonium acetate and acetic acid were obtained from Merck (Darmstadt, Germany), β-alanine from Aldrich (Steinheim, Germany), heptakis(2,6-di-*O*-methyl)-β-cyclodextrin and clenbuterol hydrochloride (Fig. 1a) from Sigma (St. Louis, MO, USA). Salbutamol hemisulfate (Fig. 1b) was kindly offered by TNO Institute (Zeist, The Netherlands).

The leading buffer was composed of 10 m*M* ammonium acetate solution adjusted to pH 2.5 with glacial acetic acid. The terminating buffer consisted of a 10 m*M* β-alanine solution adjusted to pH 2.5 with glacial acetic acid. Standard solutions of clenbuterol and salbutamol were made in 1 m*M* acetic acid. The CE buffer was composed of leading buffer containing 40 m*M* dimethyl-β-cyclodextrin as chiral selector.

2.2. Instrumentation

Counterflow transient ITP–CZE took place in a 57.5 cm×50 μm I.D. untreated fused-silica capillary (Supelco, Bellefonte, PA, USA). A programmable injection system (Prince, Lauerlabs, Emmen, The Netherlands) with pressure and voltage fine-tuning was used. Detection at 214 nm was performed using a Spectra 100 UV–Vis absorbance detector (Spectra-Physics, Mount View, CA, USA). The signal was registered on a Model 40 flat-bed recorder (Kipp and Zonen, Delft, The Netherlands).

2.3. Counterflow transient ITP procedure

The counterflow transient ITP–CZE procedure is schematically illustrated in Fig. 2. In step A, the total capillary system as well as the outlet vial is filled with leading buffer containing cyclodextrins. In step B, 173 nl of sample is hydrodynamically injected at +75 mbar for 1.6 min. This plug fills 10 cm of the capillary. Then 1 cm of terminating buffer is introduced in the capillary by 0.14 min hydrodynamic

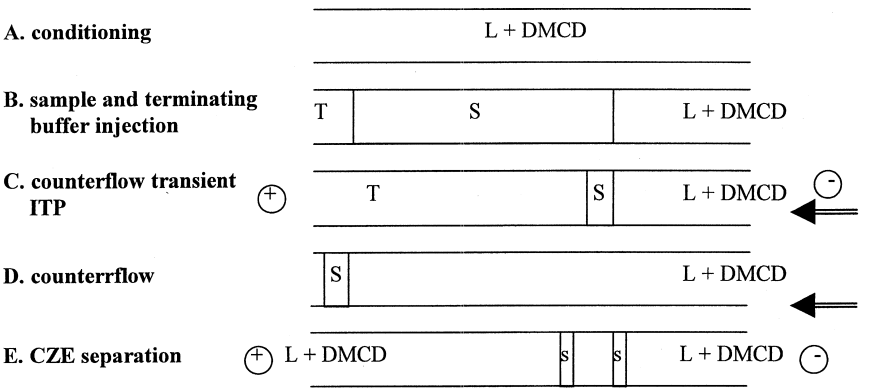

Fig. 2. Schematic representation of the counterflow transient ITP–CZE procedure. S=Sample; L=leading buffer; DMCD=dimethyl-β-cyclodextrin; T=terminating buffer.

injection at +100 mbar. In step C, counterflow transient ITP is performed during 3 min at +30 kV and −36 mbar. By this way, the sample is simultaneously focused and kept stationary in the capillary. In step D, an additional counterflow is applied for 0.78 min at −75 mbar without any voltage in order to move back the preconcentrated sample zone to the capillary inlet. Finally in step E, the terminating buffer vial is replaced with a leading buffer containing cyclodextrins vial and the CZE enantioseparation is performed at +15 kV.

3. Results and discussion

The enantioseparation of clenbuterol was first optimized using a conventional CZE–UV system. Different types of cyclodextrins were studied at several pH levels. The nature and concentration of the CZE buffer were also investigated. The best enantioseparation was performed at +15 kV with a 10 m*M* ammonium acetate buffer (pH 2.5) containing 40 m*M* dimethyl-β-cyclodextrin (Fig. 3). The enantiomeric resolution was 5.1. The migration times of salbutamol and first clenbuterol enantiomer were 18.8 and 22.8 min, respectively. The beginnings of the enantioseparation of salbutamol can be observed under those conditions.

A counterflow transient ITP step was then performed to achieve the concentration of the analytes before CZE. On one hand, the hydrodynamic velocities of the terminating buffer and the analytes in a capillary filled with CZE buffer were determined by continuous hydrodynamic injection of terminating buffer and standard solutions of analytes at +75 and +100 mbar. Precise injection volumes could thus be calculated for the method development.

On the other hand, the maximum and minimum electrophoretic velocities of the analytes during ITP and CZE were studied. Since the conductivity measured in the capillary is dependent on the volume of sample injected, its concentration and the volume of terminating buffer in the capillary, the electrophoretic velocity of the analytes is changing every time during ITP. As the sample is focused at a constant voltage, more terminating buffer is introduced in the capillary thus decreasing conductivity and slowing

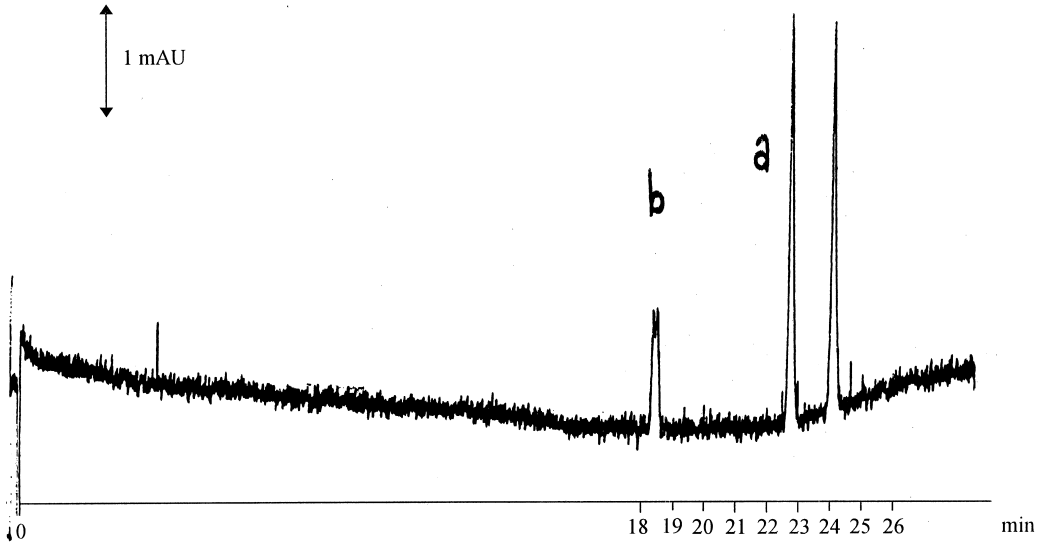

Fig. 3. Enantioseparation of clenbuterol by CZE–UV. The sample is a 10 μg/ml clenbuterol and salbutamol standard solution (=$3.6 \cdot 10^{-5}$ *M* clenbuterol). The sample is hydrodynamically injected at +75 mbar for 0.1 min. The detector range is set at 0.005 AUFS.

the analytes. Therefore, a decreasing gradient in counterpressure or, which is easier to apply, a mean between maximum and minimum values, should be applied during ITP to prevent the focused sample from moving out of the capillary inlet. The maximum velocity ($v_{e,max}$) of clenbuterol was determined using the voltage selected for the ITP step (+30 kV), in the maximum conductivity conditions, as to say a capillary filled with CE buffer (leading buffer containing cyclodextrins) and the injection of a minimum sample volume (Fig. 4). $v_{e,max}$ was 4.5 cm/min and was lower than the electrophoretic velocity of salbutamol (8.8 cm/min) which means that clenbuterol velocity should be better taken into account to prevent sample loss. The minimum velocity ($v_{e,min}$) of clenbuterol was determined applying the same voltage, in the minimum conductivity conditions which are involved at the end of ITP: the injection of a minimum sample volume and the introduction of a maximum terminating buffer volume in the capillary (Fig. 5). $v_{e,min}$ was 1.67 cm/min. According to the hydrodynamic velocities of clenbuterol in CZE and in terminating buffer, a maximum and minimum counterpressure was calculated to counterbalance $v_{e,max}$ and $v_{e,min}$. The mean of those negative pressures was applied at the capillary inlet during ITP (3 min) in order to keep the sample stationary.

The next step was to move the focused sample back to the inlet of the capillary prior to CZE. The hydrodynamic velocity of the focused sample was measured at +75 mbar in a capillary filled with

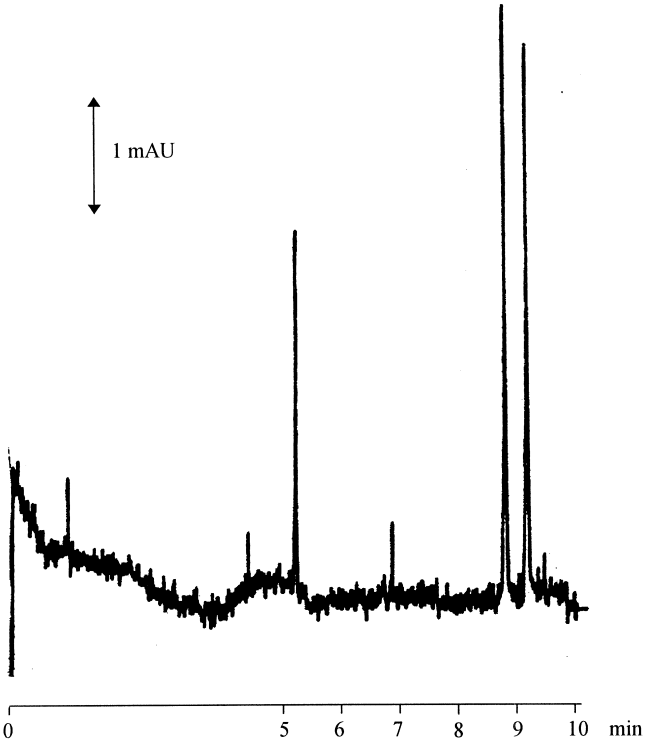

Fig. 4. Determination of the electrophoretic velocity of clenbuterol in the high conductivity buffer system. The capillary was filled with CZE buffer (leading buffer containing DMCD) and 1.25 nl of 50 μg/ml clenbuterol and salbutamol standard solution was hydrodynamically injected at +75 mbar for 0.01 min. A CZE enantioseparation was performed at +30 kV. The length of the capillary (44 cm to the detection window) divided by the migration time of the analytes in those conditions gave the maximum electrophoretic velocities of clenbuterol and salbutamol.

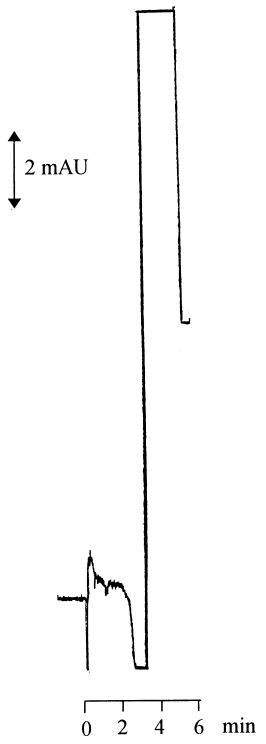

Fig. 5. Determination of the electrophoretic velocity of clenbuterol in the low conductivity buffer system. The capillary was filled with CZE buffer. 1.25 nl of 10^{-6} *M* clenbuterol and salbutamol standard solution were hydrodynamically injected at +75 mbar for 0.01 min. Then 40 cm of terminating buffer were introduced behind the sample zone by hydrodynamic injection at +100 mbar for 5.48 min and an ITP process was achieved at +30 kV without any CZE separation. The separated analytes could not be distinguished by UV detection but the front of the focused sample zone was easily detected by the drop of the baseline when the sample solvent, acetic acid, passes the detector.

terminating buffer as it fills the main part of the capillary at the end of the ITP. During focusing, the sample was concentrated into a narrow zone at the front of the sample zone so that its position in the capillary at the end of ITP was determined by the length of the injected sample plug. Therefore, the duration of the counterflow can easily be adjusted to move the zone to the capillary inlet.

Fig. 6a illustrates the enantioseparation of clenbuterol by counterflow transient ITP–CZE–UV. Improved detection sensitivity could be obtained by combining ITP sample focusing and counterflow-enhanced sample loadability. A 173-nl volume of sample was injected. The enantiomeric resolution was 4.8 indicating that the counterflow did not disturb the enantioseparation previously shown in Fig. 3. On the contrary, it extended the path length available for CE separation thus improving resolution. Clenbuterol and salbutamol migration times were 19.5 and 24.6 min, respectively and the whole process (preconcentration and enantioseparation) was achieved in 33 min.

Fig. 6b shows the enantioseparation of clenbuterol using a 10^{-6} *M* standard solution. The enantiomers could still be easily detected.

4. Conclusions

A fast in-line counterflow transient ITP–CZE–UV method has been developed for chiral analysis. It implies the use of dimethyl-β-cyclodextrin as chiral selector and the application of a hydrodynamic counterflow during the ITP step. The counterflow was performed during ITP by applying a negative pressure at the capillary inlet in order to move the concentrated sample to the inlet prior to the CZE separation. By this way, this technique allowed the in-line preconcentration and enantioseparation of clenbuterol, selected as model compound, in an extended-volume ITP–CZE system. The use of a counterflow did not disturb the enantioseparation and a resolution of 4.8 was obtained. Only five measurements of the analyte velocity and no capillary handling were required to optimize the counterpressure. The method could thus be rapidly applied to any other enantioseparation. Thanks to the extended-volume system, 173 nl of aqueous sample solution was injected without loss of enantiomeric resolution. A concentration detection limit of 10^{-6} mol/l was achieved for clenbuterol using only 3 min ITP preconcentration. This means that the detection sensitivity could be easily improved for trace analysis by increasing the ITP step. On the other hand, future research will be devoted to other detection systems, e.g., mass spectrometry, in order to improve both selectivity and detection limits.

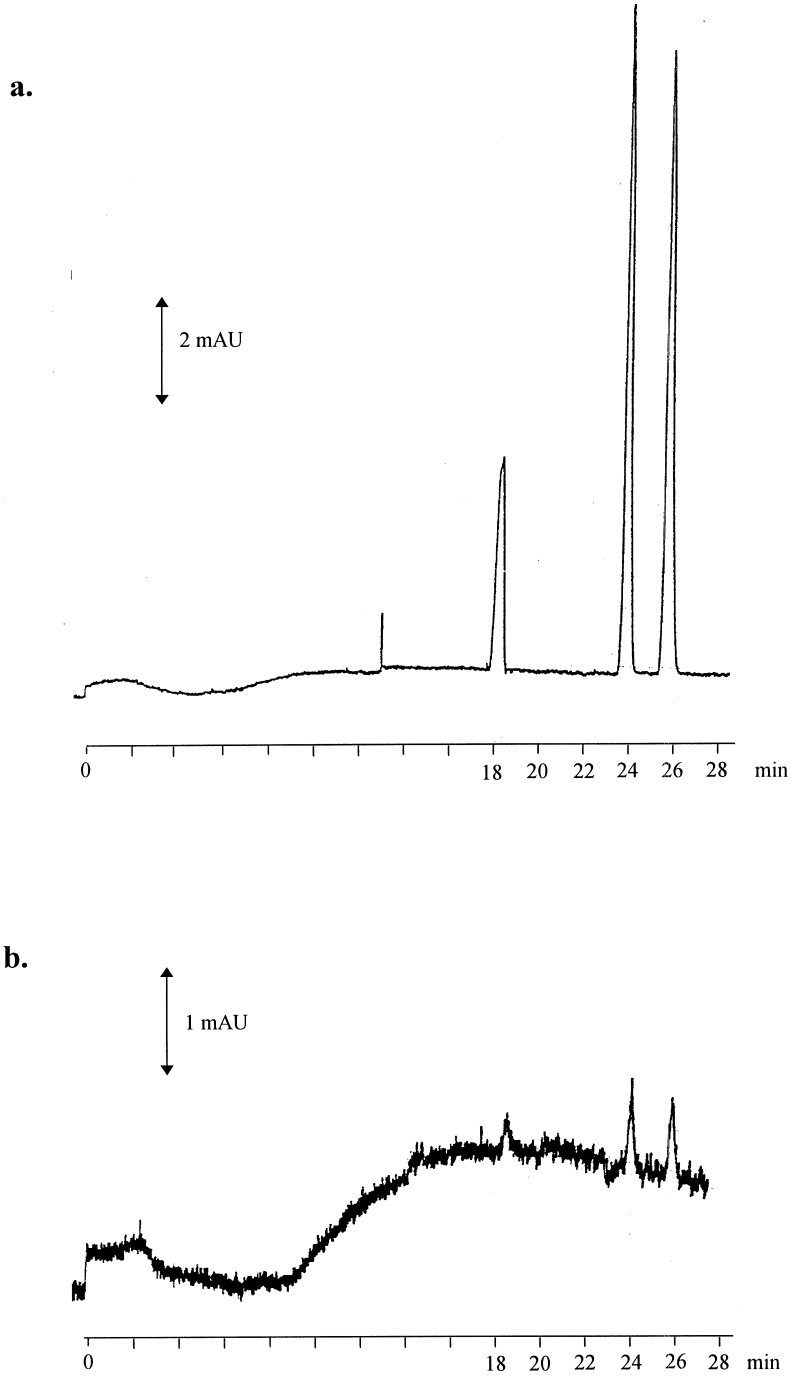

Fig. 6. Enantioseparation of clenbuterol by counterflow transient ITP–CZE–UV. (a) The sample is a 10 μg/ml clenbuterol and salbutamol standard solution (=$3.6 \cdot 10^{-5}$ *M* clenbuterol). The sample is hydrodynamically injected at +75 mbar for 1.6 min. The detector range is set at 0.01 AUFS. (b) The sample is a 10^{-6} *M* clenbuterol and salbutamol standard solution. The detector range is set at 0.005 AUFS.

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